DOCTORAL THESIS

The impact of intravesical treatments for bladder pain syndrome on normal bladder function.

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The impact of intravesical treatments for bladder pain syndrome on normal bladder function

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Abstract

Interstitial cystitis/bladder pain syndrome (IC/BPS) is characterized by pelvic pain, urinary frequency, urgency and nocturia with no evidence of bacteria or pathology. Treatment is multimodal consisting of conservative, oral, intravesical or surgical treatments for symptom control. The American Urological Association approves the intravesical use of dimethyl sulphoxide (DMSO) while it is not recommended by the European Urological Association due to insufficient evidence. Other intravesical therapies such as resiniferatoxin (RTX) and capsaicin have been trialled clinically and are not yet approved due to inconsistent results and pungency, although they could provide long-term pain relief. Therefore, this thesis aims to investigate intravesical DMSO, RTX and capsaicin and their vehicles on bladder function to address the lack of evidence surrounding DMSO and to provide possible explanations for the inconsistency and pungency associated with RTX and capsaicin.

Luminally applied DMSO, RTX and capsaicin including the respective vehicles for RTX and capsaicin (10% and 30% ethanol) were examined in pig bladders, while intravesical RTX and its vehicle (10% ethanol) were examined in mouse bladders. In pig bladders, mediator release was assessed in the urothelium/lamina propria (U/LP) during and immediately after treatment. Histological and functional studies were assessed in U/LP, detrusor and intact sections immediately after treatment. In mouse bladders, voiding behaviour was assessed before and after treatment *in vivo* while mediator release and bladder function were examined 24-hours after treatment *ex vivo*. For both models, pharmacological, mechanical and electrical stimuli were used to compare control and treated tissues.

In pig bladders, DMSO, capsaicin, 10% or 30% ethanol alterered mediator release from the U/LP while DMSO and 30% ethanol produced significant sloughing of the urothelium. DMSO enhanced U/LP muscarinic responses and detrusor nerve-mediated responses. RTX altered purinergic signalling in intact tissues and capsaicin depressed detrusor responses to muscarinic stimulation. 10% ethanol enhanced U/LP spontaneous activity while 30% ethanol depressed U/LP responses to purinergic stimulation but enhanced the responses to KCl. 30% ethanol also enhanced U/LP and detrusor responses to muscarinic stimulation and enhanced detrusor responses to nerve-mediated stimulation. In mouse bladders, RTX increased voiding frequency *in vivo*. *Ex vivo*, RTX reduced serosal acetylcholine, enhanced contractile responses to purinergic stimulation and KCl while a more substantial cholinergic component occurred

during nerve-mediated stimulation. 10% ethanol altered luminal mediator release, enhanced spontaneous activity, the contractile responses to purinergic, muscarinic and nerve-mediated stimulation.

In IC/BPS patients, the integrity of the urothelium can be significantly affected ranging from normal and intact to thin and denuded. The loss of urothelium is correlated with pain. DMSO and 30% ethanol produce many side effects in the full thickness pig urothelium in contrast to 10% ethanol, although, it produces considerable side effects in the thinner urothelium found in mouse bladders. The impact of RTX and capsaicin is minor compared to their ethanol vehicles, and alternate vehicles may reduce the side effects. DMSO appears to have no target in the bladder while RTX and capsaicin must reach nerves in the sub-urothelium to be effective. Therefore, the state of the urothelium may determine efficacy of treatment.

Declaration

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

This thesis represents my own original work towards this research degree and contains no material that has previously been submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

Katrina Smith

25th October 2018

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Publications

Abstracts as a result of this thesis

Smith, K., McDermott, C., Sellers, D., Chess-Williams, R. (2018) Effects of intravesical resiniferatoxin and ethanol on urothelial mediator release and contractile bladder responses. International Continence Society, Philadelphia 2018. Abstract available at: https://www.ics.org/2018/abstract/526

Smith, K., McDermott, C., Chess-Williams, R. (2017) Ethanol as an intravesical vehicle: effects on bladder function, Proc. ASCEPT Annual meeting, Brisbane 2017. Abstract available at: https://asceptasm.com/wp-content/uploads/2017/12/APSA-ASCEPT-poster-abstracts-51217.pdf?x82003

Smith, K., McDermott, C., Chess-Williams, R. (2017) Capsaicin: effects on detrusor and urothelial/lamina propria function. Gold Coast Health and Medical Research Conference, Gold Coast 2017. Abstract available at: https://www.goldcoast.health.qld.gov.au/research/researchers/research-week

Smith, K., McDermott, C., Chess-Williams, R. (2012) Urothelial membrane damage following the luminal application of DMSO may contribute to enhance contractile responses. Proceedings of the 4th National Symposium on Recent Advances in Urogenital and Gastrointestinal Research, Sydney 2012.

Journal articles as a result of this thesis

Smith, K.J., Chess-Williams, R., McDermott, C. (2014) Luminal DMSO: effects on detrusor and urothelial/lamina propria function. BioMed Research International, Volume 2014. Article ID 347616. Online at: http://dx.doi.org/10.1155/2014/347616

Ethics Declaration

The research associated with this thesis (chapter six) received ethics approval from the University of Queensland Animal Research Ethics Committee (Approval # Bond/150/17)

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Abbreviations

A,β-mATP: alpha,beta methylene ATP

ACh: acetylcholine

AChE: acetylcholinesterase

ADP: adenosine 5'-diphosphate

AMP: adenosine 5'-monophosphate

APF: antiproliferative factor

ASIC: acid sensing ion channel

ATP: adenosine 5'-triphosphate

Ca^{2+}: calcium

cAMP: 3'-5'-cyclic adenosine monophosphate

cGMP: guanosine 3'-5'-cyclic monophosphate

CGRP: calcitonin gene-related peptide

CNS: central nervous system

COX: cyclooxygenase

DAG: diacylglycerol

Deg: degenerin Na+ channels

DMSO: dimethyl sulphoxide

DRG: dorsal root ganglion

EFS: electrical field stimulation

EGF: epidermal growth factor

ENaC: epithelial sodium channel

EtOH: ethanol

GAG: glycosaminoglycans

HB-EGF: heparin-binding EGF

IC/BPS: interstitial cystitis/bladder pain syndrome

IP3: inositol triphosphate

K^+: potassium

KCl: potassium chloride

LDH: lactate dehydrogenase

L-NNA: L-N^G-Nitroarginine

NA: noradrenaline

NA^+: sodium

NGF: nerve growth factor

NK: neurokinin

NO: nitric oxide

NOS: nitric oxide synthase

PACAP: pituitary adenylate cyclase

PAR: Proteinase-activated receptor-2

PG: prostaglandin

PKC: protein kinase C

PLC: phospholipase C

ROCK: Rho-associated protein kinase I and II

RTX: resiniferatoxin

SEM: standard error of the mean

SP: substance P

THP: Tamm-Horsfall protein

TNF: tumour necrosis factor

Trk: tyrosine kinase receptor

TRP: transient receptor potential

TTX: tetrodotoxin

UDIF: urothelially derived inhibitory factor

UDP: uridine 5'-diphosphate

UP: uroplakin

UTP: uridine 5'-triphosphate

vACHt: vesicular acetylcholine transporters

Chapter 1:
General introduction

1.1 The bladder

Gross anatomy

The bladder is an extraperitoneal hollow muscular organ that acts as a temporary storage vessel for urine (Fry, 2013). The empty bladder is shaped like a tetrahedron (three-sided pyramid) consisting of an apex, a base, a superior surface and two inferolateral surfaces. Superiorly it is covered with pelvic peritoneum on which the small intestine and the sigmoid loop lie. In the female, the anteverted uterus lies against the posterosuperior surface. Anteriorly, the bladder is situated behind the pubic symphysis, and the apex is attached to the umbilicus by the median umbilical ligament (a remnant of the fetal urachus). Laterally, the inferolateral surfaces meet the obturator internus muscle above supported by the levator ani muscle below. Only the uppermost surface of the triangular base is covered by visceral peritoneum that forms the retro-vesical pouch in men and the utero-vesical pouch in women. The posterior surface relates to the rectum, vas deferens and seminal vesicles in males and the anterior vagina and upper part of the cervix in females. The base additionally receives its ureters at the upper lateral angles (Ellis, 2005, Mangera et al., 2013).

Where the inferolateral surfaces meet the base, the muscle fibres converge into a funnel-like structure at the base known as the bladder neck or internal sphincter. Further down, the male bladder neck fuses with the prostate gland, and in females, the bladder neck lies in the connective tissue of the anterior vaginal wall forming the urethra. The male's urethra is about 20cm long and passes along the length of the penis before emptying. The female urethra is much shorter, being only about 4cm long and opens to the outside just after passing through the external urethral sphincter. At the apex of the prostate in males and midway along the urethra in the female is the external sphincter **(Figure 1.1),** (Yang, 2003, Mundy, 2004).

Internally, two ureteral orifices and the bladder outlet form the borders of a triangular region called the trigone. The trigone is both histologically and embryologically different from the rest of the bladder containing a rich plexus of neuronal tissue that is firmly attached to the underlying muscle tissue **(Figure 1.2)**, (Roosen et al., 2009, Mangera et al., 2013).

The bladder can be divided into two principal areas that are the bladder body and the bladder base. The *body* is located above the ureteral orifices, and the *base* consists of the trigone, the bladder outlet, deep detrusor and the anterior bladder wall **(Figure 1.2)**,(Mangera et al., 2013).

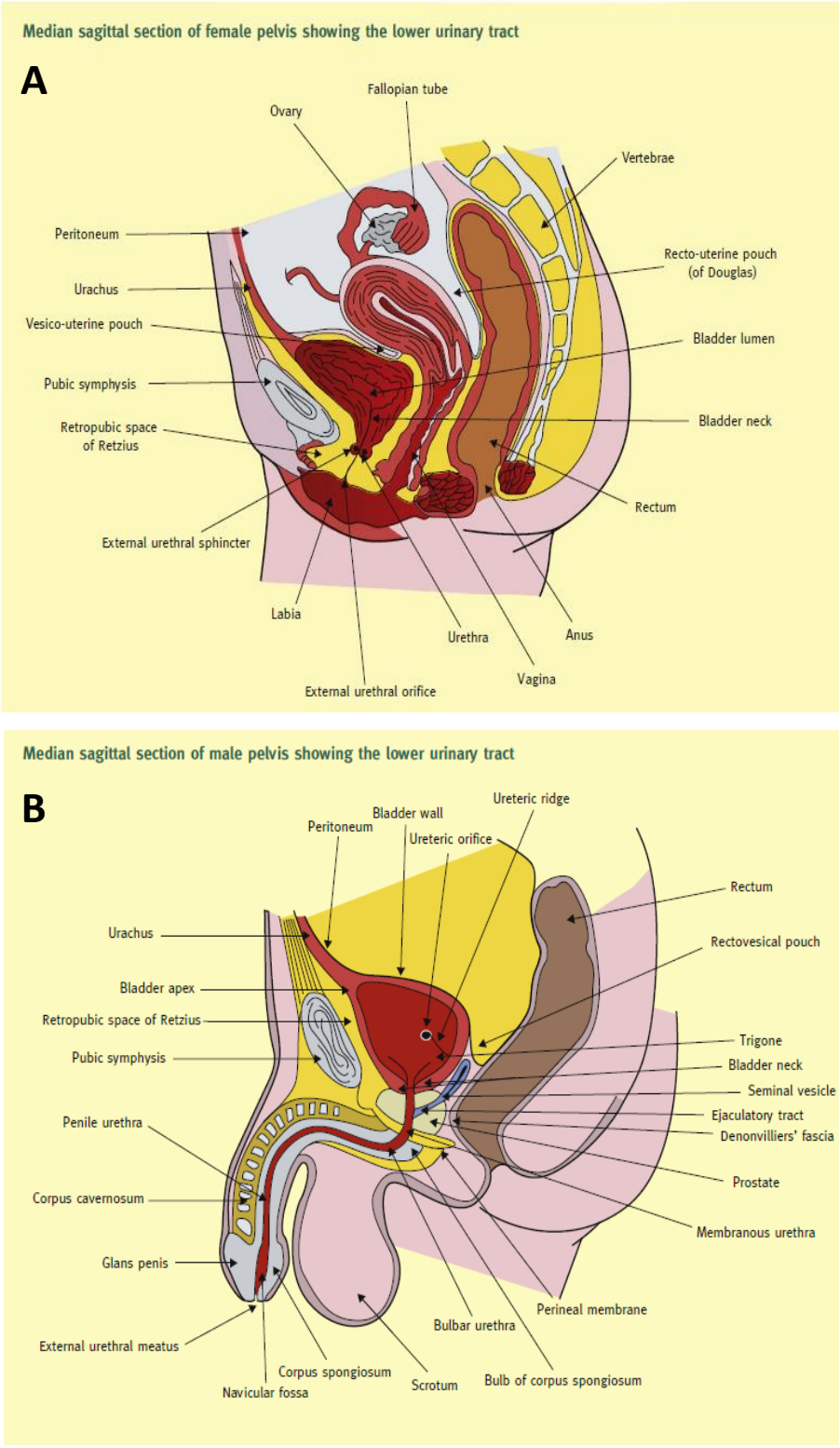


Figure 1.1: A and B represent the anatomical locations of both female and male bladders and surrounding structures (reproduced with permission from the publisher, (Mangera et al., 2013)).

1.2 The layers of the bladder wall

There are four distinct layers of the bladder that include the serosa, the detrusor muscle (smooth muscle), the sub-urothelial lamina propria and the urothelium **(Figure 1.3A)**. The serosa is the outermost adventitial layer composed of two fascial layers. The first layer covers and supports the anterior and lateral aspect of the bladder and the second layer is present on the body and posterior aspects of the bladder. Both tissues fuse to complete the serosa (Mundy, 2004, Mangera et al., 2013).

The detrusor

The detrusor is the smooth muscle layer of the bladder that accounts for most of the thickness of this organ. The detrusor found in the bladder body, sometimes referred to as the dome, consists of an interlacing meshwork of randomly orientated muscle fibres that are responsible for the storage and pump functions of the bladder (Elbadawi, 1996, Mundy, 2004, Mangera et al., 2013). It is only at the bladder base near the bladder outlet that the detrusor muscle fibres organise themselves into three distinct layers. The inner and outer layers are comprised of longitudinal muscle while the middle layer consists of circumferential muscle that forms the internal sphincter (Elbadawi, 1996, Turner and Brading, 1997, Mundy, 2004, Mangera et al., 2013).

Further down the urethra, the external sphincter is also composed of three distinct layers. The outer layer consists of striated muscle that is a part of the pelvic floor musculature known as the pubo-urethral sling. The middle layer is composed of striated muscle that lies within the urethra wall, and the innermost layer consists of urethral smooth muscle (Mundy, 2004).

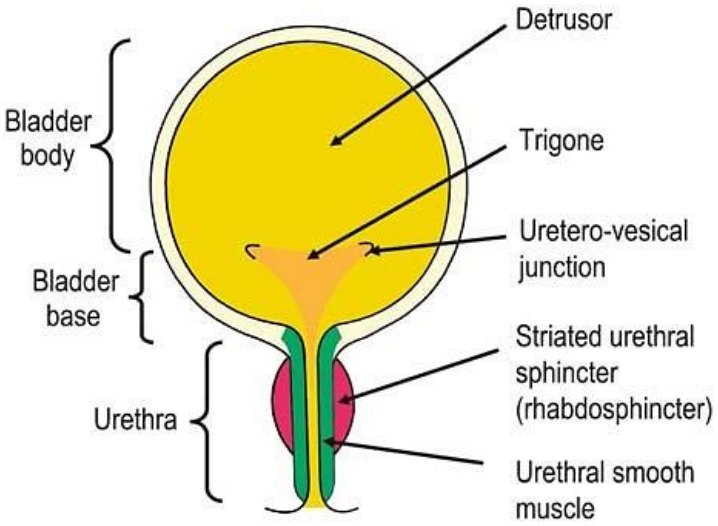


Figure 1.2: Key areas of the bladder including the locations of the trigone, external sphincter, bladder body and bladder base (reproduced with permission from the publisher, (Westerling and Andersson, 2007)).

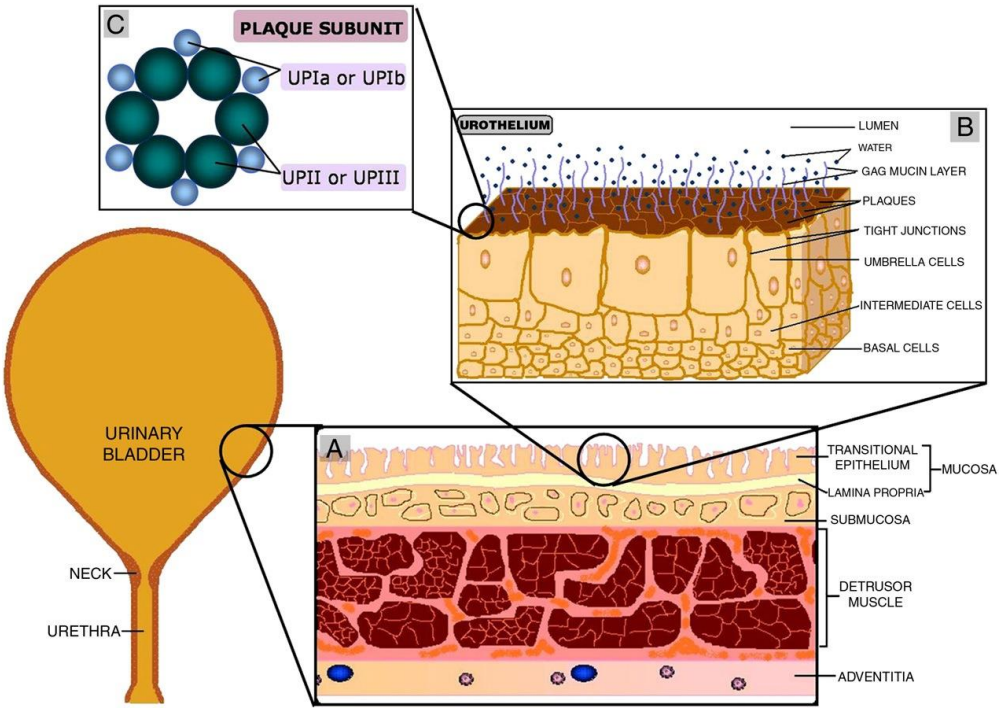


Figure 1.3: (A) Represents a histological illustration of the layers of the bladder. (B) Identifies the morphology of the urothelial layer including the secreted GAG layer. (C) Illustrates the arrangement of umbrella cell uroplakins (reproduced with permission from the publisher, (GuhaSarkar and Banerjee, 2010)).

The sub-urothelium/lamina propria

Internally adjacent to the detrusor muscle lies the sub-urothelium/lamina propria that connects the detrusor to the urothelium and is considered to be part of the signalling system between

both structures **(Figure 1.3A)**, (Andersson and McCloskey, 2014). It consists of loose connective tissue containing nerve fibres, fibroblasts, blood capillaries, smooth muscle cells (muscularis mucosae) and interstitial cells (Aitken and Bagli, 2009, Dixon and Gosling, 1983, Wiseman et al., 2003).

Interstitial cells

In humans and other species including the pig, the interstitial cells are a distinct cellular population found directly beneath the urothelium, in the deeper lamina propria, and within the detrusor both inter and intramuscularly (Steiner et al., 2018). In the lamina propria in humans, interstitial cells have also been associated with the nerves and vascular smooth muscle (Johnston et al., 2010, Andersson and McCloskey, 2014).

Interstitial cells of the bladder are similar to interstitial cells of the gut and are typically described as having a spindle type morphology with several branches originating from a central soma (Davidson and McCloskey, 2005a, Kubota et al., 2011). Both the interstitial cells of the gut and bladder stain for Kit (tyrosine kinase receptor) and vimentin (structural protein) whereas smooth muscle cells nearby do not. However, it is not certain how many other structures in the bladder express Kit and therefore the use of this marker is controversial (Hirst and Ward, 2003, Davidson and McCloskey, 2005b, Kubota et al., 2011, Steiner et al., 2018). In the bladder, vimentin and Kit do not label the same population of interstitial cells as Kit-positive cells are a subpopulation of vimentin-positive cells. Moreover, some interstitial cells that were Kit or vimentin positive have been labelled with other antibodies such as platelet-derived growth factor receptor alpha which confirms other sub-types exist within the bladder (McCloskey, 2013, Kanai et al., 2014, Neuhaus et al., 2018).

The interstitial cells of the gut act as “pacemaker” cells and transmit signals from the enteric nerves to the smooth muscle (Hirst and Ward, 2003). A similar “pacemaker” role of interstitial cells has also been proposed in the bladder. However, this theory is debatable (Shafik et al., 2004, Hashitani et al., 2004)

Due to their close approximation to nerves in the lamina propria, interstitial cells in this region are thought to sense bladder filling, modulate afferent signals and amplify signals from the urothelium to the detrusor (Johnston et al., 2010, Birder et al., 2012, Wiseman et al., 2003,

Steiner et al., 2018).In the human lamina propria and using 3D-ultrastructural analysis, Neuhaus et al. (2018) identified fibroblast, myoid (smooth-muscle-like), and branching-type interstitial cells. The myoid-type interstitial cells were flat sheet-like cells that stacked in parallel to the urothelium and were interconnected by gap junction protein Cx43. It was further suggested by Neuhaus et al. (2018) that the myoid-type interstitial cells play a role in the afferent signalling cascade.

Gap junctions allow communication between cells and are made up of membrane proteins called connexins that form as pores to allow the passage of ions and low weight metabolites between the cytosol of connected cells. Five sub-groups of connexins exist which are α, β, γ, δ, or ε. However, connexins are named on their predicted molecular weight, for example, Cx43 is approximately 43 kD in size. Several types of connexins with different properties exist. In mammals, Cx43 is the most abundantly expressed and studied connexin (Steinberg, 1998, Nielsen et al., 2012).

In the detrusor, two types of interstitial cells have been described by ultrastructural analysis using transmission electron microscopy which were the fibroblast and myoid type that were arranged in a dense plexus both outside and inside muscle fascicles that make no connection to nerves. The myoid-type interstitial cells in the detrusor were suggested to be "sensors" of stretch during bladder filling (Rasmussen et al., 2009).

The urothelium

The urothelium is the innermost layer that functions as a barrier and interface between the lumen of the bladder and its underlying tissues. It is composed of three distinct cellular layers that consist of an innermost basal layer attached to a basement membrane, an intermediate layer and an apical layer of large hexagonal cells termed umbrella cells **(Figure 1.3B)**, (Martin, 1972, Jost et al., 1989, Veranic et al., 2004). The urothelium is known to have one of the slowest cycling epithelia in the human body and is considered to be far more effective as a barrier than the epidermis (Hicks, 1975). However, urothelial injury or pathology can rapidly increase turnover (Martin, 1972, Hicks, 1975, Birder and Andersson, 2013). Most of the urinary tract is lined with urothelium, but there are distinct differences observed in the ureters that contain fewer uroplakins, and the proximal urethra where the urothelium transforms into stratified columnar epithelium with microvilli (Liang et al., 2005, Romih et al., 2005).

The basal and intermediate layer

Urothelial cellular differentiation originates from the basal layer upwards with increasing complexity in the arrangement and structure of cells. The basal cell layer lies immediately adjacent to a basement membrane. The cells are in a single layer that ranges from 5-10 μm in size. These cells have a steady but low-level proliferation rate that gives rise to the intermediate layer. The intermediate layer consists of 3-4 layers of cells that are approximately 20 μm in size. Intermediate cells induce proliferation when the urothelium has been injured **(Figure 1.3B)** (Baskin et al., 1997, Tash et al., 2001).

The umbrella cells

The hexagonal umbrella cells form the urine-contacting layer of the urothelium. Umbrella cells are continuously exposed to mechanical forces such as stretch and shear stress. Their size can range from 50-120 μm in diameter depending on the degree of bladder expansion. These cells are joined together by tight junctions that are crucial for maintaining urothelial permeability **(Figure 1.3B**) (Tash et al., 2001, Carattino et al., 2013).

Two unique characteristics of umbrella cells are the sub-apical trajectorial network of cytokeratins and plaques composed of protein particles known as uroplakins that cover the apical surface which are also found in fusiform vesicles in the cytoplasm (Veranič and Jezernik, 2002, Truschel et al., 2002, Veranic et al., 2004). Four major uroplakins have been identified in humans which are UPIa, UPIb, UPII and UPIII (Olsburgh et al., 2003, Matuszewski et al., 2016). Several isoforms of UPIII exist including UPIIIa, UPIIIb and the newly discovered UPIIIc (Desalle et al., 2014). Uroplakins occupy 90% of the apical surface of the cell making it a lot thicker than the basolateral membrane, therefore, the apical surface of the cell is known as the asymmetric unit membrane **(Figure 1.3C)** (Porter et al., 1967, Veranic et al., 2004, Zhou et al., 2012).

The association of umbrella cell and uroplakins is consistent throughout the entire urinary tract, albeit in smaller quantities in the urethra, and they are thought to play a role in barrier permeability functions and cellular membrane organization such as the reversible adjustments of the apical surface area (Hicks, 1975, Truschel et al., 2002, Kong et al., 2004, Hu et al., 2002, Liang et al., 2005). In rabbit bladders, UPIII found in the fusiform vesicles beneath the surface of umbrella cells was found in larger amounts on the apical surface in response to stretch by

insertion of these vesicles in the membrane increasing the surface area (Truschel et al., 2002). The accumulation of uroplakin containing fusiform vesicles in the umbrella cells indicates that these cells are involved with uroplakin synthesis and trafficking to the apical surface. Subsequent research has identified that the apical directing protein, myelin and lymphocyte protein was associated with uroplakin positive vesicles (Zhou et al., 2012). In addition, uroplakin UPIa is the receptor site for the establishment of pathogenic *E. coli* that is responsible for urinary tract infections (Zhou et al., 2001). Furthermore, urothelial tumours including the surrounding unaffected urothelial tissue can have altered uroplakin expression in contrast to normal healthy urothelium which may reflect the high recurrence rate of this condition (Zupancic and Romih, 2013). As uroplakins are markers of fully differentiated urothelial cells, it was also shown that UPIII was present on lung cancer material and was related to metastatic urothelial cancer (Gruver et al., 2012).

In addition to the unique characteristics of umbrella cells that contribute to the permeability barrier of the urothelium, the cell surface also expresses a superficial layer of glycosaminoglycan's for additional surface fortification (Soler et al., 2008).

The glycosaminoglycan layers

The glycosaminoglycan (GAG) layer of the urothelium consists of negatively charged sulphated GAGs bound to a negatively charged protein core forming a proteoglycan. The GAG layer binds water into gel creating a buffer between urine and the urothelial cell membrane **(Figure 1.3B)** (Janssen et al., 2013). The GAG layer of the urothelium is the first line of defence against the adhesion of bacteria, including solutes and toxic substances in the urine (Parsons et al., 1979, Parsons et al., 1990, Lilly and Parsons, 1990, Poggi et al., 2000). GAG's have also been found in the basal membrane of the urothelium and have been suspected to play a role in urothelial proliferation, barrier maintenance, cell signalling or urothelial adhesion (Janssen et al., 2013).

The relatively impermeable barrier of the urothelial layer makes diffusion of drugs into the bladder wall to treat damaged areas or tumours a challenge (GuhaSarkar and Banerjee, 2010). However, disruption to the urothelial GAG layer can occur due to infection, radiation/oncology drugs or interstitial cystitis/bladder pain syndrome (IC/BPS), producing a cascade of events that result in leakage of urine through the urothelium to the underlying tissue causing pain and hypersensitivity (Parsons et al., 1994, Parsons et al., 1998, Parsons et al., 2001).

1.3 Function and innervation of the bladder

Function

The bladder has two primary functions; that is to act as a low-pressure reservoir for urine and to generate sufficient pressure to expel urine (Mangera et al., 2013). The kidneys produce urine that is transported to the bladder via the ureters. The adult bladder can usually accommodate around 500 ml of urine without any significant increase in pressure. The micturition cycle of storing and expelling urine is a complex system of reflexes involving the bladder, the internal and external sphincter, the urethra, the peripheral nerves, spinal cord and brain (Yang, 2003, Mangera et al., 2013). Most of the micturition cycle is spent in the storage phase in which the bladder is required to relax and fill while only a short period of the cycle is spent in actual voiding (de Groat and Yoshimura, 2001, Andersson and Arner, 2004).

Bladder nerves are divided into autonomic efferent innervation (parasympathetic and sympathetic pathways), sensory afferent innervation and somatic motor innervation. These nerves differ in function, anatomy and chemistry and will be described below. It should be noted that in general, bladder nerve supply and connectivity is similar across species. (Keast et al., 2015)

The parasympathetic pathway

When the volume of urine collected by the bladder reaches micturition threshold, afferent activity from the bladder initiates a voiding reflex by stimulation of the parasympathetic pathway which is also inhibitory to the sympathetic and somatic pathways (de Groat et al., 2015). Parasympathetic outflow is considered to provide the major excitatory output to the urinary bladder to activate and perpetuate detrusor muscle contractions while relaxing the urethra and bladder outlet. The cholinergic pre-ganglionic neurons are found in the intermediolateral region of the sacral spinal that exit via the anterior roots S2-S4. Their axons travel via the pelvic nerve to the pelvic plexus and bladder wall (de Groat, 2006, Birder et al., 2010a). Evidence suggests that the majority of nerves found in the detrusor are cholinergic (Ek et al., 1977, de Groat et al., 2015).

Parasympathetic stimulation requires the pre-ganglionic axons to release acetylcholine (ACh) at the post-ganglionic synaptic junction activating nicotinic N2 receptors. Nicotinic receptors

are present on the sympathetic and parasympathetic ganglia and play an integral role in the control of bladder function by mediating fast synaptic transmission (Degroat and Saum, 1976, Yoshimura and de Groat, 1997, de Groat et al., 2015). Nicotinic transmission at the synapse can be further modulated by mechanisms involving muscarinic, purinergic, adrenergic and enkephalinergic receptors (Tobin and Sjögren, 1995, Santicioli et al., 1983, Degroat and Kawatani, 1989).

The post-ganglionic parasympathetic neurons provide the excitatory input to the detrusor by releasing ACh at the neuromuscular junction that acts on the muscarinic receptors inducing contraction (Mantegazza and Naimzada, 1967, Chess-Williams et al., 2001, de Groat et al., 2015). Along with ACh, adenosine 5'-triphosphate (ATP) is co-released from the parasympathetic nerves where it acts on purinergic receptors and plays a motor role in the detrusor (Mundy, 2004, Kumar et al., 2007). Parasympathetic innervation of the urethra releases nitric oxide (NO) that acts in the urethra relaxing the smooth muscle to permit the expulsion of urine (de Groat et al., 2015).

Acetylcholine and cholinergic receptors

Acetylcholine from parasympathetic nerves is released from neural varicosities at synapse junctions between neurons and the neuromuscular junction initiating coordinated and sustained bladder contraction (Mundy, 2004, de Groat et al., 2015). Early studies identified the immediate contraction of the guinea pig bladder in response to exogenous ACh (Mantegazza and Naimzada, 1967). In the synaptic cleft, ACh is broken down to acetate and choline by acetylcholinesterase, and the choline is taken up into the nerve terminal by Na^+ mediated secondary transport. It is unknown if these breakdown products have any physiological effects (Mundy, 2004).

Muscarinic receptors are classified into five subtypes (M1-M5). The bladder is known to contain all five muscarinic receptor subtypes at the mRNA level. Generally, M1, M3 and M5 receptors predominately couple to G_q proteins activating phospholipase C and M2 and M4 receptors couple to G_i proteins that inhibit adenyl cyclase thereby decreasing 3'-5'- cyclic adenosine monophosphate (cAMP) (Caulfield, 1993, Caulfield and Birdsall, 1998, Sigala et al., 2002).

Muscarinic receptors have been found pre-synaptically on parasympathetic nerve terminals innervating the bladder. M1 appears to enhance post-ganglionic ACh release during high-frequency nerve firing, and M2/M4 may inhibit post-ganglionic ACh release (Somogyi and de Groat, 1992, Somogyi et al., 1994, D'Agostino et al., 2000). Muscarinic receptors are expressed throughout the bladder, although, the M2 and M3 subtypes are more commonly expressed within the detrusor of animals and humans (Maeda et al., 1988, Yamaguchi et al., 1996, Mimata et al., 1997). The M2 receptor is predominantly expressed in comparison to M3 and the M2:M3 ratio is approximately 3:1 in human, rabbit, guinea pig and pig detrusor and 9:1 in the rat detrusor (Wang et al., 1995, Yamanishi et al., 2000). In the rabbit detrusor, the density of muscarinic receptors was found to be higher in the body when compared to the base of the bladder (Levin et al., 1980). Wang et al., (1995) demonstrated coupling of the M2 and M3 receptor to G-Proteins, specifically G_i and G_q in human and rat bladders. This was followed by antagonist studies on the rat, rabbit and human detrusor that identified the M3 receptor responsible for initiating and driving contraction of the bladder (Chess-Williams et al., 2001, Fetscher et al., 2002, Schneider et al., 2004a). The source of calcium (Ca^{2+}) that initiates contraction in response to muscarinic stimulation comes from both extracellular and intracellular sources. Thus, reductions in contraction have been noted via L-type Ca^{2+} channel antagonism, to blockade of IP3 induced release but not by prevention of Ca^{2+} induced Ca^{2+} release by antagonizing the ryanodine receptors (Schneider et al., 2004a, Schneider et al., 2004b, Wuest et al., 2007). Furthermore, some antimuscarinic drugs have inhibitory effects on the L-type channels in the bladder demonstrating their coupling to muscarinic receptors (Wuest et al., 2005, Frazier et al., 2008).

M2 receptor contributions

Although M2 receptors are the most populous in the detrusor, their exact role has not yet been fully clarified. In the bladder, it has been hypothesized that M2 receptors may inhibit relaxation by coupling to G_i protein opposing sympathetically mediated increases in cAMP (Caulfield, 1993, Hegde and Eglen, 1999, Andersson, 2004). Studies involving human, rabbit and guinea pig bladder tissue have all noted a decrease in adenylate cyclase activity in response to muscarinic agonists (Ruggieri et al., 1987, Noronha-Blob et al., 1989). The sympathetic nervous system, stimulating adenylate cyclase promoting the formation of cAMP that subsequently relaxes the detrusor will be discussed in greater detail below (Rang, 2007, Hayashi et al., 2016).

Studies by Ehlert et al. (2005), (2007) were unable to demonstrate a direct contractile response from M2 receptors in response to agonists but instead were able to demonstrate an indirect response. Drugs exhibiting an M2 agonist profile inhibited the effects of relaxing agents (i.e., forskolin, isoproterenol) allowing contraction to occur in M3 knock out (KO) mice or mice with experimentally inactivated M3 receptors. *In vivo* studies based on the rat bladder have shown some involvement of the M2 receptor with sympathetic β-adrenoceptor activity as the inhibitory potency of M2 antagonist methoctramine decreased after pre-treating the tissues with the β-adrenoceptor antagonist propranolol but not with M3 antagonist darifenacin (Hegde et al., 1997). The M2 receptor may become more relevant in pathological conditions as there are some reports of increased M2 receptor populations and function in the bladders of spinal cord injured animals (Braverman and Ruggieri, 2003, Pontari et al., 2004, Matsumoto et al., 2012).

Smooth muscle contractile mechanisms

Contraction of the detrusor initiated by muscarinic receptor stimulation results in the influx of extracellular Ca^{2+} and an increase of intracellular Ca^{2+} by the sarcoplasmic reticulum (SR) within the cell. The release of intracellular Ca^{2+} is triggered via a G_q protein-coupled mechanism involving the breakdown of the membrane phospholipid phosphatidylinositol by the enzyme phospholipase C. The breakdown products are inositol triphosphate (IP3) and diacylglycerol (DAG). Diacylglycerol remains associated with the cell membrane and is metabolized rapidly. It has two main functions: further cleavage to arachidonic acid, a precursor for prostaglandin production; and activation of protein kinase C which modulates ion channel function, in particular, Ca^{2+} channels. This results in Ca^{2+} influx contributing to the response. Inositol triphosphate binds to specific receptors on the SR releasing Ca^{2+} into the sarcoplasm. To induce contraction, Ca^{2+} binds to calmodulin forming a Ca^{2+}/calmodulin complex that activates myosin via myosin light chain kinase (MLCK). The MLCK catalyses the phosphorylation of myosin resulting in cross-bridge cycling thus initiating a contraction **(Figure 1.4)**. Smooth muscle relaxation occurs in response to falling levels of Ca^{2+} that triggers myosin phosphatase (MLCP) to dephosphorylate the myosin light chain. Calcium re-uptake occurs via an ATP Ca^{2+} pump on the SR (CaATPase) while some is additionally lost across the cell membrane restoring basal resting conditions (Bayliss et al., 1999, Fry et al., 2002, Andersson and Arner, 2004, Mundy, 2004).

Myogenic activity is defined as the ability of a smooth muscle cell to generate contractile activity with no external stimuli. The detrusor muscle cells are believed to be electrically coupled by gap junctions. In the normal bladder, the extensive coupling between smooth muscle bundles does not occur as each smooth muscle cell couples to only a few of its adjacent cells (Brading, 1997, Neuhaus et al., 2002). The synchronous activity that occurs during contraction, for example, is mostly perpetuated by dense innervation and direct nerve activation. However, in the case of an unstable bladder of neurogenic and idiopathic origin including outflow obstruction, an increase in detrusor coupling has been proposed (Brading, 1997). Immunohistochemistry has identified the presence of Cx43 and Cx45 in the human detrusor and in the case of neurogenic bladder dysfunction due to spinal cord injury and patients with urge incontinence, Cx43 was shown to be up-regulated compared to control stable bladders (John et al., 2003, Haferkamp et al., 2004, Neuhaus et al., 2005). Additionally, gap junctions may contribute to detrusor tone, and more recently, Cx43 has been suggested to be an important biomarker for some metastasizing bladder cancers (Wang et al., 2001, Zhang et al., 2016).

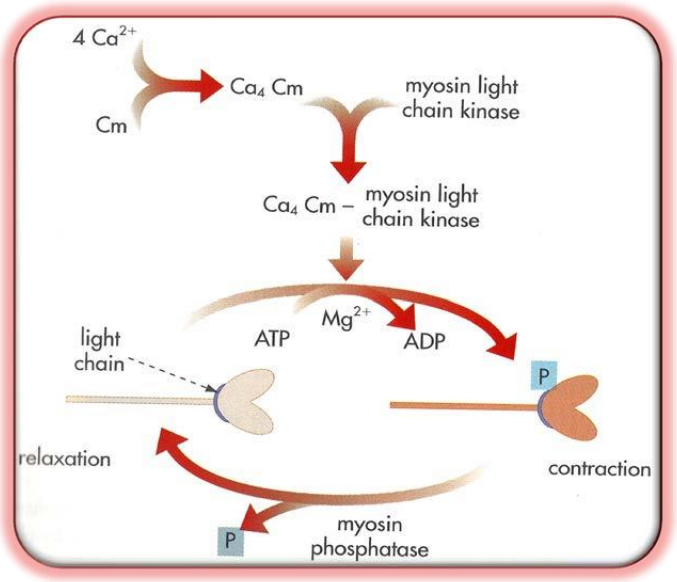


Figure 1.4: The cellular cascade of reactions that are involved with smooth muscle contraction and relaxation in the detrusor. Cm=Calmodulin (reproduced with permission from the publisher,(Mundy, 2004)).

Calcium sensitization: PKC/ROCK

Increases in intracellular Ca^{2+} initiate bladder smooth muscle contraction. However, increases in intracellular Ca^{2+} do not always reflect the magnitude of contraction. This mechanism is

termed calcium sensitization, and both protein kinase C (PKC) and RhoA/Rho-associated kinase (ROCK) pathways are involved (Takahashi et al., 2004, Durlu-Kandilci and Brading, 2006, Hayashi et al., 2016).

The isoforms of ROCK, ROCK-I and ROCK-II are activated by RhoA that sets in motion Ca^{2+} sensitization that inhibits smooth muscle MLCP by phosphorylation of the MYPT1 subunit (Kimura et al., 1996). Similarly, PKC activated by DAG after stimulation of PLC phosphorylates the inhibitory protein CPI-17 of MLCP (Frazier et al., 2008, Zhang and Disanto, 2011). ROCK has also been implicated in the phosphorylation of CPI-17, and when it has been phosphorylated, CPI-17 has a much greater affinity for MLCP that potentiates contraction. (Eto et al., 1995, Koyama et al., 2000). The protein expression of RhoA, ROCK-I, ROCK-II and CPI-17 has been found in human and rat bladders (Wibberley et al., 2003, Takahashi et al., 2004).

In vitro, contractions of the human detrusor to carbachol (muscarinic agonist) are inhibited in a concentration-dependent manner by ROCK inhibitors. (Schneider et al., 2004a, Kirschstein et al., 2014). However, no attenuation of carbachol-induced contractions with PKC inhibitors has been observed indicating the PKC pathway is not essential for enhancing detrusor contraction (Schneider et al., 2004a).

In the human detrusor, using selective antagonists for the M2 and M3 receptor, Shahab et al (2012) discovered that both M2 and M3 receptors contribute to the ROCK and PKC Ca^{2+} sensitization in the presence of carbachol, Ca^{2+} and guanosine 5'-triphosphate (GTP), however, M3 was the predominant subtype. They have suggested that the M2 receptor indirectly influences the ROCK/PKC pathway by downregulation of cAMP. However, it was found more recently in the same conditions that cAMP inhibited the ROCK pathway but not the PKC pathway (Hayashi et al., 2016).

The ROCK/PKC pathway not only involves muscarinic receptors in the bladder but extends to other signalling pathways in the bladder that have been reviewed elsewhere (Peters et al., 2006, Zhang and Disanto, 2011, Hypolite and Malykhina, 2015).

ATP and purinergic receptors

In many species, the bladder's response to nerve stimulation is not entirely prevented by muscarinic receptor blockade. Burnstock et al. (1972) suggested that this non-adrenergic, non-

cholinergic (NANC) response is due to ATP that is co-released with ACh from parasympathetic terminals (Dumsday, 1971). Generally, parasympathetic stimulated detrusor contractions in healthy human bladders are predominantly mediated by cholinergic transmission with little or no contribution by non-cholinergic mechanisms such as ATP (Sibley, 1984, Kumar et al., 2004). However, this is not always the case with aging bladders, some types of human bladder dysfunction and normal bladder contractile activity in animals that all display a degree of atropine resistance to nerve-mediated responses (Burnstock et al., 1972, Bayliss et al., 1999, Yoshida et al., 2001). Overall, ATP can affect both motor and sensory function within animal and human bladders despite the absence of atropine-resistant contractions in healthy human tissue (Inoue and Brading, 1991, O'Reilly et al., 2002, Birder et al., 2003, Birder et al., 2004, Kumar et al., 2004, Burnstock, 2014). Adenosine 5'-triphosphate is promptly broken down by ecto-nucleotidases to form adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenosine which interact with P2X, P2Y and adenosine receptors in the bladder (Edwards and Gibb, 1993, King et al., 1997, Robson et al., 2006, Burnstock, 2014).

P2X Receptors

There are seven P2X receptors (P2X1 -P2X7) which are ligand-gated ion channels that open in response to the binding of extracellular ATP (Ralevic and Burnstock, 1998, Ruggieri, 2006, Burnstock, 2018). There is evidence that these receptors have the potential to co-assemble and form heteromeric channels that exhibit distinct pharmacological and regulatory properties compared to their homomultimer form such as P2X2/3, P2X4/6, P2X1/4 (North, 2002, Lazarowski et al., 2003, Kaan et al., 2010).

Immunohistochemistry studies have identified P2X1-7 receptors in the human bladder. In general, P2X1 is the predominant receptor expressed in the detrusor and to a lesser extent, P2X2 and P2X7 (Elneil et al., 2001, O'Reilly et al., 2002, Svennersten et al., 2015). The P2X7 receptors have been associated with immunity and the inflammatory response (Burnstock, 2000, Moncao-Ribeiro et al., 2014, Geraghty et al., 2017).

Purinergic contractile mechanisms

The P2X1 receptor has been implicated as the receptor responsible for contributing to the motor component of bladder function. The general theory of how this transpires is that parasympathetic stimulation concomitantly releases ACh and ATP in which ATP binds to the

P2X1 receptors resulting in an influx of sodium (Na^+). Sodium carries most of the charge with some contributions from Ca^{2+} entry as P2X1 channels are relatively non-selective to cations. The resulting depolarization from the inward flux, in turn, opens L-type Ca^{2+} channels initiating a more substantial Ca^{2+} inflow. Within the cell, the increasing levels of intracellular Ca^{2+} are supplemented by Ca^{2+} release from the SR which trigger a contraction **(Figure 1.5)** (Mundy, 2004).

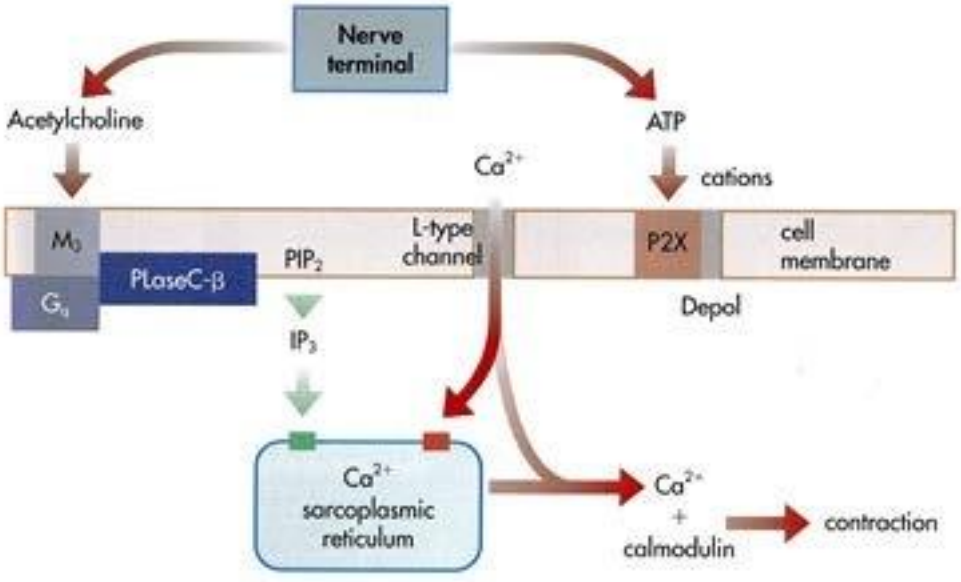


Figure 1.5: Putting it all together: The cellular cascade of events that occurs within the detrusor smooth muscle cell when ACh and ATP are co-released resulting in a contraction (reproduced with permission from the publisher, (Mundy, 2004)).

P2Y Receptors

To date, there are eight subtypes of P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14). The P2Y family can be categorized into a subfamily in which P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 predominately couple to G_q activating phospholipase C and P2Y12, P2Y13, P2Y14 couple to G_i proteins inhibiting adenylate cyclase and decreasing cAMP (Ralevic and Burnstock, 1998, Lazarowski et al., 2003, Burnstock, 2018). Nucleotides and di-nucleotides such as uridine 5'-triphosphate (UTP), ADP, and uridine 5'-diphosphate (UDP) bind to and activate P2Y receptors (Abbracchio et al., 2006). Little is known of the distribution of P2Y receptors in the human detrusor. However, in animal models, there is some evidence of functional P2Y receptors in the bladder. While ATP may play a role in contracting the detrusor at the start of micturition, activation of the P2Y receptors may contribute to relaxation

of the bladder neck and may also play a role modulating bladder tone (Tong et al., 1997, Obara et al., 1998, Aronsson et al., 2010, Yu et al., 2013, Burnstock, 2014).

Nitric oxide

Nitric oxide (NO) is another transmitter released from the parasympathetic nerves and it plays a significant role in the relaxation of the trigone, bladder neck and urethra during micturition as well as decreasing intravesical pressure during bladder filling. However, the influence of NO on the detrusor is minor when compared to the actions on the bladder outlet (Andersson et al., 1983, Persson et al., 1992, Persson and Andersson, 1992, Smet et al., 1996).

In contrast to other transmitters that are stored and released in vesicles, NO is not stored but is synthesized immediately before release. Nitric oxide is produced from the conversion of the amino acid L-arginine to L- citrulline by nitric oxide synthase (NOS) which requires co-factor NADPH. Nitric oxide diffuses across the target cell membrane binding to soluble guanylate cyclase which converts guanosine triphosphate (GTP) into 3,5 cyclic guanine monophosphate (cGMP). Increased levels of cGMP inhibit Ca^{2+} entry into the cell, activate potassium (K^{+}) channels hyperpolarizing the cell and stimulate cGMP–dependent protein kinase leading to cellular relaxation (Mundy, 2004). Many cell types within the bladder have displayed cGMP immunoreactivity following exposure to NO, including the urothelium, interstitial cells, detrusor smooth muscle fibres, bladder neurons and nerve terminals (Smet et al., 1996).

Three isoforms of NOS have been identified which include neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The prefix for all three NOS isoforms denotes the origin of purification and cloning. However, all three isoforms can be found in numerous mammalian tissues (Michel and Feron, 1997). Both nNOS and eNOS are modulated by intracellular Ca^{2+} and produce low levels of NO for a short duration. Nitric oxide at low concentrations is a signalling molecule that participates in smooth muscle relaxation, blood flow and neurotransmission. Inducible NOS, on the other hand, is produced by a Ca^{2+} independent pathway and is synthesized in copious amounts for a longer duration (hours to days) in inflammatory conditions. (Nussler and Billiar, 1993, Bian et al., 2012, Vannini et al., 2015). In the rat bladder, it was found that iNOS in response to bladder outlet obstruction promoted the increase in capacity, spontaneous bladder contractions and fibrosis associated with this condition (Felsen et al., 2003).

The cellular targets within the bladder of neuronally released NO are unclear. Its inhibitory actions on the trigone, bladder neck and urethra may indicate the presence of a functional mechanism on the muscles that are within this region or NO may modulate neurotransmitter release from peripheral nerves (Mundy, 2004). Other transmitters associated with the relaxation of the bladder neck include vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), 5-hydroxytryptamine (5-HT), ATP (presumably on P2Y receptors) and adenosine (Hills et al., 1984, McMurray et al., 1998, Hernandez et al., 2006).

The sympathetic pathway

The role of the sympathetic nervous system is to allow for the accumulation of urine at low pressures while increasing bladder outlet resistance during the storage phase of the micturition cycle (Vera and Nadelhaft, 1992, Ochodnicky et al., 2013).

The sympathetic pre-ganglionic neurons arise from the intermediolateral region of the lower thoracic and upper lumbar spinal cord segments T11-L2, making synaptic connections with post-ganglionic neurons in lumbosacral sympathetic chain ganglia (SCG), the inferior mesenteric ganglia (IMG) and pelvic plexus. These nerves then go on to provide input to the bladder mostly via the hypogastric nerve and partly via the pelvic and pudendal nerves (de Groat, 2006, Bortolini et al., 2014, de Groat et al., 2015).

Sympathetic innervation is mostly found at the trigone, bladder neck, urethra and blood vessels of the bladder although clear differences in species exist. In contrast to the parasympathetic nerves, sympathetic supply to the human detrusor is very rare and appears to be restricted to the vesicourethral junction and around autonomically innervated blood vessels in the sub-urothelium and detrusor. In males, sympathetic supply is enhanced in the pre-prostatic urethra forming a plexus that runs parallel to the smooth muscle cells. In the female proximal urethra, sympathetic supply is very sparse, and cholinergic nerves preferentially innervate the smooth muscle (Gosling et al., 1977, Kluck, 1980, Vera and Nadelhaft, 1992).

There are three classes of adrenoceptors which are differentiated as α_1, α_2 and β. All three classes of adrenoceptors are G-protein coupled and are expressed throughout the human bladder (Michel and Vrydag, 2006, Rang, 2007, Moro et al., 2013, Alexander, 2017).

Like the parasympathetic pathway, sympathetic pre-ganglionic fibres release ACh activating the postsynaptic N2 nicotinic receptors that initiate the next corresponding action potential (de Groat et al., 2015). Sympathetic input can additionally extend to the parasympathetic ganglia where it can modify the parasympathetic output via α_1 and α_2 adrenoceptors (Degroat and Saum, 1976, Keast et al., 1990). At the neuromuscular junction, the post-ganglionic fibres release noradrenaline that activates α-adrenoceptors found at the bladder base promoting urethral tone, while β-adrenoceptors found in the bladder body promote relaxation of the detrusor as the bladder fills **(Figure 1.6)**, (Degroat and Saum, 1976, Larsen, 1979, Kunisawa et al., 1985, Bagot and Chess-Williams, 2006).

The α-adrenoceptors that maintain urethral tone

The α_1 and α_2-adrenoceptors can be further classified as ($\alpha_{1A,}$ $\alpha_{1A/L,}$ α_{1B} and α_{1D}) and (α_{2A}, $\alpha_{2B,}$ and α_{2C}) respectively (Alexander, 2017). The α_1-adrenoceptor couples to a G_q protein that activates phospholipase C and the α_2-adrenoceptor is coupled to a G_i protein that inhibits adenylate cyclase (Bylund, 1988, Docherty, 1998, Nishimune et al., 2010). Moreover, it also was found that the contraction of the isolated sheep urethra mediated by noradrenaline was dependent on both extracellular and intracellular sources of Ca^{2+} (Garcia-Pascual et al., 1991). It was later found that the source of Ca^{2+} influx is via the L-type channels in response to activation of the α_1-adrenoceptor in dog and rabbit urethra (Testa et al., 1993, Zhong and Minneman, 1999).

Binding experiments and pharmacological studies of the human and rabbit bladder base have identified a predominance of the α_1-adrenoceptors in comparison to the α_2-adrenoceptors (Kunisawa et al., 1985, Levin et al., 1988). To determine the specific α_1-adrenoceptors subtype that mediates the contractile response of the urethra, the binding of prazosin (α_1-adrenoceptor antagonist) after irreversible alkylation of the other α_1-adrenoceptors, apart from the α_{1A} subtype with chloroethylclonidine, identified that the α_{1A}-adrenoceptor was the most populous in the male prostatic urethra smooth muscle (Testa et al., 1993). This finding is consistent with mRNA expression and in situ hybridization analysis of the human male and female proximal urethra (Nasu et al., 1998). In addition to the α_{1A}-adrenoceptor, the $\alpha_{1A/L}$-adrenoceptor which has been demonstrated to have originated from the α_{1A}-adrenoceptor gene has a low affinity for prazosin and has also been suggested to contribute to smooth muscle contraction of the urethra (Taniguchi et al., 1997, Gray et al., 2008). Pharmacological studies of the circular

smooth muscle in the pig urethra and human bladder neck identified the predominant involvement of the $\alpha_{1A/L}$-adrenoceptor mediating the contractile response to noradrenaline (Bagot and Chess-Williams, 2006, Yoshiki et al., 2013).

β-adrenoceptors

The β-adrenoceptors are classified into three subtypes (β_1- β_3). These receptors promote relaxation of the detrusor and decreased intra-urethral pressure promoting the storage function of the bladder (Rao et al., 1980, Bortolini et al., 2014). Generally, β-adrenoceptors are coupled to G_s proteins and act by stimulating adenylate cyclase promoting the formation of cAMP which has been shown to relax the human detrusor (Rang, 2007, Hayashi et al., 2016). Bladder β-adrenoceptors have additionally been implicated in opposing the contractile activity of muscarinic receptors, may also influence K^+ channels and some downstream Rho-kinase signalling contributing to bladder relaxation. (Ferro, 2006, Frazier et al., 2008, Michel and Sand, 2009, Klausner et al., 2009, Cernecka et al., 2015, Hayashi et al., 2016).

In the human bladder, mRNA for all three subtypes has been detected, however, determining actual protein expression of β subtypes has been difficult, hampered by a lack of suitable tools (Igawa et al., 1999, Michel and Vrydag, 2006). Based on pharmacological studies, detrusor relaxation in humans is mostly mediated by β_3-adrenoceptors. Although, it is different for other species such as rats for example, where both β_2 and β_3-adrenoceptors are involved (Yamazaki et al., 1998, Wuest et al., 2009, Michel et al., 2011, Propping et al., 2013).

Somatic innervation

The somatic motor component arises from Onufs nucleus that is located in the lateral ventral horn in the S2 and S4 segments of the spinal cord. These nerve fibres travel with the preganglionic parasympathetic fibres and send their axons into the pudendal nerve where it separates and enters the sacral plexus. The somatic nerves innervate the sphincter and pelvic floor musculature via cholinergic nicotinic receptors (skeletal muscular type N1) causing contraction and promoting continence **(Figure 1.6)**. The combined activities of the sympathetic and somatic pathway increase bladder outlet resistance (Mundy, 2004, Seth et al., 2013).

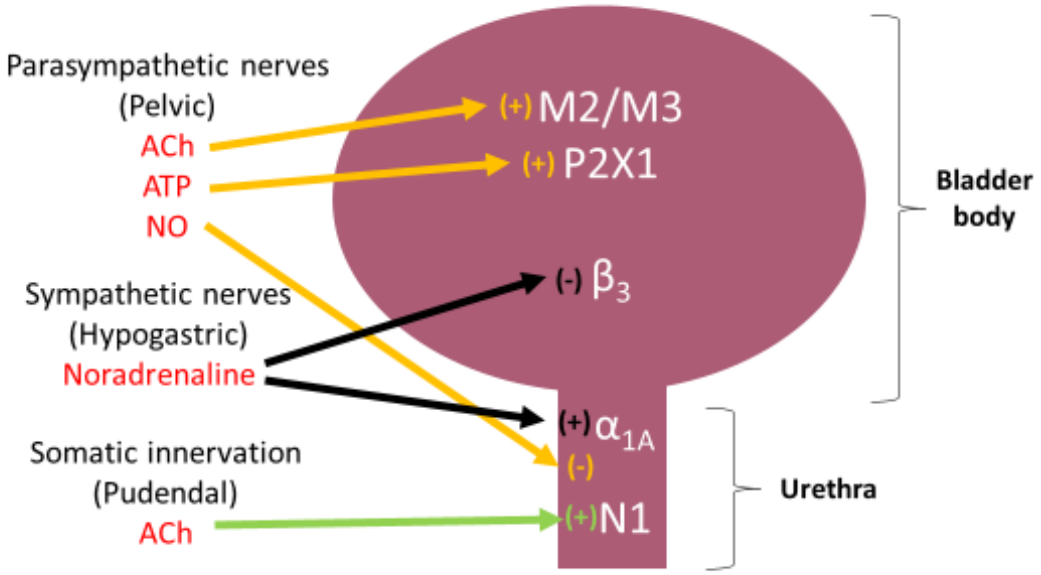


Figure 1.6: A summarized illustration of the parasympathetic, sympathetic and somatic innervation of the urinary bladder including their respective transmitters and their action on receptor targets.

Sensory afferent innervation

Although equally important as the efferent pathway, the afferent pathway is less understood. Afferent neurons relay sensory information from the bladder to the spinal cord transferring signals to higher autonomic centres in the brainstem. From the brainstem, sensory information is projected to the various coordinating centres for bladder function that determines parasympathetic, sympathetic and somatic output (Andersson, 2002, Yoshimura, 1999, Kanai and Andersson, 2010).

Bladder afferents originate from neuronal cell bodies in the dorsal root ganglia (DRG) that lie close to each side of the spinal cord. Afferent signals leave the bladder and urethra and travel via afferent axons found in the pelvic and hypogastric nerves (Janig and Morrison, 1986, Habler et al., 1990, de Groat et al., 2015). Both the pelvic and hypogastric afferent pathways have been implicated with sensations of normal bladder filling and bladder pain, whereas the pelvic afferent pathways have been associated with the sensation of impending micturition and urethral thermal sensations (Janig and Morrison, 1986, Janig and Koltzenburg, 1990, Andersson, 2002).

In both humans and animals, afferent nerves are found in four major regions of the bladder which include the detrusor smooth muscle bundles, beneath the urothelium forming a sub-

urothelial plexus, within the urothelium and associated with blood vessels. The afferent fibres found in the sub-urothelial plexus are relatively sparse in the dome of the bladder but become gradually denser approaching the bladder neck with prominent innervation seen in the trigone (Gabella and Davis, 1998, Wakabayashi et al., 1993, Smet et al., 1997, Andersson, 2002, de Groat and Yoshimura, 2009).

There are two main types of bladder afferent nerves which are small myelinated Aδ-fibres that have conduction velocities of >2.5m/s and small un-myelinated C-fibres that have conduction velocities of <2.5m/s (Vera and Nadelhaft, 1990, de Groat et al., 2015). Furthermore, bladder afferent nerves can be divided into three separate functional populations: mechanoreceptors, chemoreceptors and silent afferents (Habler et al., 1990, Shea et al., 2000, Habler et al., 1988). The Aδ-fibres and C-fibres reportedly contain both mechanosensitive and chemosensitive properties (de Groat, 2006, Habler et al., 1990).

Afferent mechanoreceptors

Mechanoreceptors are made up of both low and high threshold fibres indicating that a population of both Aδ and C-fibres are mechanosensitive (Bahns et al., 1987, Habler et al., 1990, Shea et al., 2000, Habler et al., 1993). Low threshold fibres are associated with normal non-painful micturition and pressures between 5-15 mmHg, pressures when humans report the first sensations of bladder filling. High threshold fibres appear to be involved with painful sensations generated by over 30mmHg when most humans report pain and discomfort (de Groat and Yoshimura, 2009). The small population of C-fibres that have been found to be mechanosensitive do not respond to distension at normal pressures but are activated when distention pressures reach 30-50 mmHg whereas the Aδ-fibres are associated with normal low physiological pressures (Habler et al., 1990, Habler et al., 1993). Overall, it is thought that C-fibres are not essential for normal voiding, although, they become more significant in bladder pathology (de Groat et al., 2015).

Studies on the afferent nerves in the rat bladder by Shea et al. (2000) demonstrated that in addition to mechanoreceptors, which display a linear increase in discharge frequency as the bladder slowly fills, other receptors had firing rates that plateaued or decreased with increasing pressure. These receptors have been interpreted to be volume receptors.

Afferent chemoreceptors

Chemoreceptors sensitive to chemical irritation of the urothelium have been mostly associated with C-fibres (Andersson, 2002). C-fibres are sensitive to neurotoxins, capsaicin, resiniferatoxin, tachykinins, NO, ATP, prostaglandins, endothelin's and neurotrophic factors which reportedly modulate afferent responses (Habler et al., 1990, de Groat, 2006, de Groat et al., 2015). Aδ-fibres may also possess chemoreceptive properties, as they can become more mechanosensitive in response to chemical irritants (Janig and Koltzenburg, 1990, Wyndaele, 2010).

Silent afferents

Silent afferents are unresponsive to distension and chemical stimuli. (Habler et al., 1990, Shea et al., 2000) Although in the cat, a small subpopulation of "silent" afferents became mechanosensitive during inflammatory conditions and may, therefore, play a more active role in pathological conditions (Habler et al., 1988).

Afferent neurotransmission

Activation of the bladder afferent nerves in response to filling is proposed to occur by a direct and indirect pathway. The *direct* mechanism relies on mechanically gated ion channels that are expressed on the afferent terminals associated with the detrusor that are activated by mechanical stretch in response to bladder filling. These are most likely to be ENaC/ASIC/degerin Na^+ channels and transient receptor potential (TRP) channels (Zagorodnyuk et al., 2009a). The *indirect* mechanism relies on interplay with chemical mediators that are either released from the detrusor or urothelium such as ATP, NO that interact with afferent receptors associated with the urothelium such as P2X receptors, etc. The urothelium and associated transmitters and receptors will be discussed in greater detail below (Sun et al., 2010, Zagorodnyuk et al., 2009a). Not only do afferent nerves transmit impulses from the bladder to the CNS, there is evidence that these nerves also possess an "efferent" function and can modify bladder behaviour by releasing transmitters into the peripheral tissues (Maggi et al., 1984, Maggi, 1990, Ishizuka et al., 1995a, Templeman et al., 2003).

Afferent nerve transmitters and receptors

Bladder afferent nerves contain various neuropeptides and receptors that play a role in transduction. Studies have identified neuropeptides associated with bladder afferent nerves such as substance P (SP), calcitonin gene-related peptide (CGRP) pituitary-adenyl cyclase activating polypeptide (PACAP), leucine enkephalin, corticotropin-releasing factor, vasoactive intestinal polypeptide (VIP) growth-associated protein 43, nitric oxide synthase (NOS) glutamic acid and aspartic acid (de Groat and Yoshimura, 2009, de Groat et al., 2015). Peptide containing afferent fibres containing CGRP, SP, NKA, and VIP are found throughout the human bladder but are more abundant in the sub-urothelial layers (Wakabayashi et al., 1993, Smet et al., 1997). Substance P and CGRP are predominantly associated with C -fibres which can mediate an inflammatory response when released (Yoshimura and de Groat, 1997, Andersson, 2002, Birder et al., 2002, de Groat and Yoshimura, 2009). Afferent neurons express many receptors associated with sensory mechanisms. The more frequently studied ones include transient receptor potential (TRP) receptors, purinoceptors, and neurokinin receptors (Brechenmacher et al., 1998, de Groat et al., 2015, Grundy et al., 2018b)

Transient receptor potential channels

The transient receptor potential (TRP) channel superfamily has mostly been implicated in sensory signal detection and transduction from the peripheral nervous system to the central nervous system. Stimuli include sensing changes in temperature, chemicals, touch, light, sound, local environment changes and osmolality (Venkatachalam and Montell, 2007, Mickle et al., 2015). So far twenty-eight members have been identified comprising of six subfamilies, which are TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystin) and TRPML (mucolipin) (Clapham, 2003, Avelino et al., 2013).

Throughout the bladder, TRPV1, TRPV2, TRPV4, TRPA1 and TRPM8 channels are highly expressed on sensory afferent nerve fibres in humans and rodents (Avelino and Cruz, 2006, Birder, 2007, Andersson et al., 2010, Avelino et al., 2013). Evidence suggests that members of the TRP family play essential roles in bladder function and sensory perception and respond to a diversity of stimuli such as mechanical force, osmotic stress, heat and chemical compounds as well as having important roles in physiology and pathology (Clapham, 2003, Dinis et al., 2004, Coelho et al., 2015).

In the bladder, function and expression of TRPV1, 2 and 4 channels are well documented with TRPV1 being the most investigated channel (Andersson et al., 2010, Avelino et al., 2013). Both the TRPV1 and TRPA1 channels are currently under investigation as targets for chronic pain disorders and bladder pathology while much less is known about TRPM8 (Birder, 2007, Furuta et al., 2012).

The TRPV1 channels

Transient receptor potential vanilloid 1 is a non-selective ion channel with high Ca^{2+} permeability allowing the influx of cations (mostly Ca^{2+}) upon activation. It is activated by capsaicin, heat (43 °C) and low pH at physiological temperatures. Repeated exposure to capsaicin induces TRPV1 receptor desensitization with a loss of neuropeptides and degeneration of capsaicin-sensitive afferent nerve terminals (Wharton et al., 1986, Caterina et al., 1997, Tominaga and Caterina, 2004, Tominaga and Tominaga, 2005).

Immunohistochemistry studies in the human bladder found TRPV1 channels associated with unmyelinated nerve fibres running beneath the urothelium and with nerve endings entering the urothelium that terminates beneath the superficial cells. Transient receptor potential vanilloid 1 channels were also found in the endothelium of capillaries and arterioles, irregularly in the detrusor, in mast cells located in the sub-urothelium and near blood vessels (Lazzeri et al., 2004b). Furthermore, Ost et al. (2002) found TRPV1 receptors on both Aδ and C-fibres with a dense network of sub-urothelial C-fibres containing TRPV1 channels.

The role of TRPV1 channels in normal bladder function is not fully understood, but due to their sub-urothelial/urothelial location, they may be receptive to intravesical stimuli (Lazzeri et al., 2004b). In TRPV1 KO mice, the conscious voiding behaviour was reduced in duration and volume with an increase in non-voiding contractions which suggests that the TRPV1 receptors contribute to stability in the storage phase in non-pathological conditions. These mice also display depressed afferent responses and mediator release from the urothelium (Birder et al., 2002, Yoshiyama et al., 2015, Grundy et al., 2018b). Activation of TRPV1 channels in the rat bladder with TRPV1 agonists (capsaicin and resiniferatoxin) produces a contractile response reportedly mediated by the release of neuropeptides (Maggi, 1990, Saitoh et al., 2007). Also, in mice, the TRPV1 channel has been found essential for pain and heat perception (Caterina et al., 2000, Tsubota et al., 2018). Furthermore, the TRPV1 channel appears to play a more prominent role in bladder overactivity and pain associated with inflammation with no contribution to the initiation of inflammation (Charrua et al., 2007, Mingin et al., 2015).

Purinoreceptors

The P2X3 and P2X2/3 receptors are predominantly located on both Aδ and C-fibres where they respond to ATP. It is believed that urothelially released ATP facilitates bladder mechanosensory transduction sensing volume changes, conveying that information back to the CNS (Ford and Cockayne, 2011, Burnstock, 2014).

Neurokinin receptors

Neurokinin (NK) receptors interact with neuropeptides such as SP, neurokinin A (NKA) and neurokinin B (NKB). There are three classes of NK receptors (NK1, NK2 and NK3) which are all G-protein coupled (Nakanishi, 1991, Maggi, 1995a). Although NK receptors can be found throughout the bladder, there is pharmacological evidence of NK1, NK2 and NK3 receptors on rat DRG neurons that were found to have elevated intracellular Ca^{2+} when activated by agonists (Brechenmacher et al., 1998, Burcher et al., 2000, Bie and Zhao, 2011). Immunohistochemistry of rat DRG neurons revealed that the NK1 receptor was expressed on both A and C-fibres and was often co-expressed with the TRPV1 channels (Zhang et al., 2007).

The ligand for NK receptors, SP can be found at the site of inflammation, and noxious stimuli can promote its release (Liu et al., 1997, Van Der Kleij and Bienenstock, 2007). During an induced inflammatory state, a larger proportion of rat DRG neurons were responsive to SP and displayed diminished desensitization when compared to non-inflammatory conditions (Bie and Zhao, 2011). Thus, the presence of NK receptors on the afferent nerves and their activation by SP allows for delivery of nociceptive information back to the CNS.

Within the bladder, SP and NKA have been found to promote detrusor contraction via the NK2 receptors (Uckert et al., 2002, Templeman et al., 2003). Additionally, the NK2 receptor in the bladder may contribute to modulation of spontaneous activity and bladder tone (Grundy et al., 2018a).

Prostanoids

Prostanoids are a subclass of signalling molecules that include prostaglandins, prostacyclins and thromboxanes (Rahnama'i et al., 2012b). These signalling molecules are produced and released by many mammalian cells where they employ a variety of actions in tissues and cells. The most common actions are contraction and relaxation in smooth muscle. Prostanoids can also moderate neuronal activity by either stimulating or inhibiting neurotransmitters and sensitizing sensory fibres to noxious stimuli (Narumiya et al., 1999).

Prostanoids are mentioned because their presence in the bladder is associated with modulation of the afferent and efferent neural responses, distension, mechanical trauma, increased osmolarity, ATP release, inflammation and products of bacterial metabolism (Collier et al., 1975, Farkas et al., 1980, Husted et al., 1980, Brown and Burnstock, 1981, Jeremy et al., 1984, Jeremy et al., 1986, Dveksler et al., 1989, Aizawa et al., 2010a).

Cyclooxygenase (COX) enzymes are the rate-limiting step in prostanoid synthesis. Two COX enzymes have been identified which are aptly named COX 1 and COX 2. Cyclooxygenase 1 is found in the majority of cells and regulates physiological processes. COX 2 expression is induced by growth factors, pro-inflammatory cytokines and tumour promoters (Andersson, 2002, Cao and Prescott, 2002). The expression of COX 1 in the guinea pig bladder has been found in the intermediate and basal layers of the urothelium, associated with a population of interstitial cells in the lower lamina propria and on the surface of detrusor muscle bundles (de Jongh et al., 2007, de Jongh et al., 2009).

The primary prostanoids are prostaglandin (PG)D_2, PGE_2, $PGF_{2\alpha}$ PGI_2 and thromboxane (TX)A_2 which are produced in different amounts in the bladder depending on the species (Narumiya et al., 1999, Rahnama'i et al., 2012b). However, the human bladder produces prostanoids in the following order PGI_2>PGE_2>$PGF_{2\alpha}$>TXA_2 (Jeremy et al., 1987). Each prostanoid interacts with a specific G-protein coupled receptor. Prostaglandin I2 is the ligand for IP receptors, PGE_2 interacts with EP receptors ($EP_{1\text{-}4}$), $PGF_{2\alpha}$ binds to FP receptors and TXA_2 is the ligand for TP receptors (Breyer et al., 2001). These receptors have been found in the bladder except the FP_2 receptor which is specific for $PGF_{2\alpha}$ although there is pharmacological evidence for FP and TP receptors in the rabbit detrusor (Collins et al., 2009, Rahnama'i et al., 2012b).

In non-pathological conditions, prostanoids in the bladder contribute to nerve-mediated detrusor contractions, act as amplifiers of afferent input while the bladder is filling as well as maintenance of bladder tone and spontaneous activity (Bultitude et al., 1976, Klarskov, 1987, Collins et al., 2009, Aizawa et al., 2010b). Prostanoids are also highly expressed in the bladder in inflammatory and pathological conditions (Farkas et al., 1980, Wheeler et al., 2001, Kim et al., 2006). Proteinase-activated receptor-2 (PAR2) is a G-protein coupled receptor that is involved in the pain response and inflammation (Bao et al., 2014). It was recently found in the rat bladder, activation of the PAR2 receptor produces referred hypersensitivity in the lower abdomen which was attenuated by indomethacin (COX inhibitor) and a TPPV1 channel

antagonist suggesting that prostanoids (most likely PGE_2) are potentiated by TRPV1 activation and contribute to pain by sensitizing nociceptors (Tsubota et al., 2018). Overall, prostanoids in the bladder can modify bladder activity directly by receptors on the detrusor and indirectly by neuromodulation (Dobrek and Thor, 2015).

1.4 The intelligent urothelium

In addition to its role as a barrier between urine and the underlying tissue, the urothelium has now been distinguished as a sophisticated functional and sensory transduction unit capable of communicating intra-luminal changes to the underlying tissues (Birder, 2010). This communication is made possible due to the expression of various receptors/ion channels and chemical mediators that are typically associated with sensory neurons (Sun and Chai, 2002, Aizawa et al., 2011a, Dunning-Davies et al., 2013, Grundy et al., 2018b, Sellers et al., 2018).

There are many receptors and ion channels expressed on the urothelium that include purinergic (P2X and P2Y) receptors, cholinergic (muscarinic and nicotinic) receptors, adrenoceptors (both α and ß), protease-activated receptors, Na^+ channels (Enac and the Deg/Enac family), trkA receptors, p75 receptors, cannabinoid receptors (CB1, CB2), neurokinin receptors (NK1, NK2), PGE_2 receptors (EP1, EP2), angiotensin II receptors (AT1), endothelin receptors (ETA, ETB) big K^+ channels (BK) and 5-hydroxytryptamine receptors (5HT). This enables the urothelium to respond to a variety of sensory outputs from the urine and other structures of the bladder. Urothelial cells are also capable of secreting ATP, ACh, prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$ PGI_2), NO, ciliary neurotrophic factor (CNF), macrophage migration inhibitory factor (MIF), leukocyte inhibitory factor (LIF), hydrogen sulphide (H2S), urothelium-derived inhibitory factor (UDIF), and interleukins (IL) that can modulate bladder activity **(Figure: 1.7)** (Birder, 2010, Sellers et al., 2018). The more well-known mediators and receptors will be discussed below.

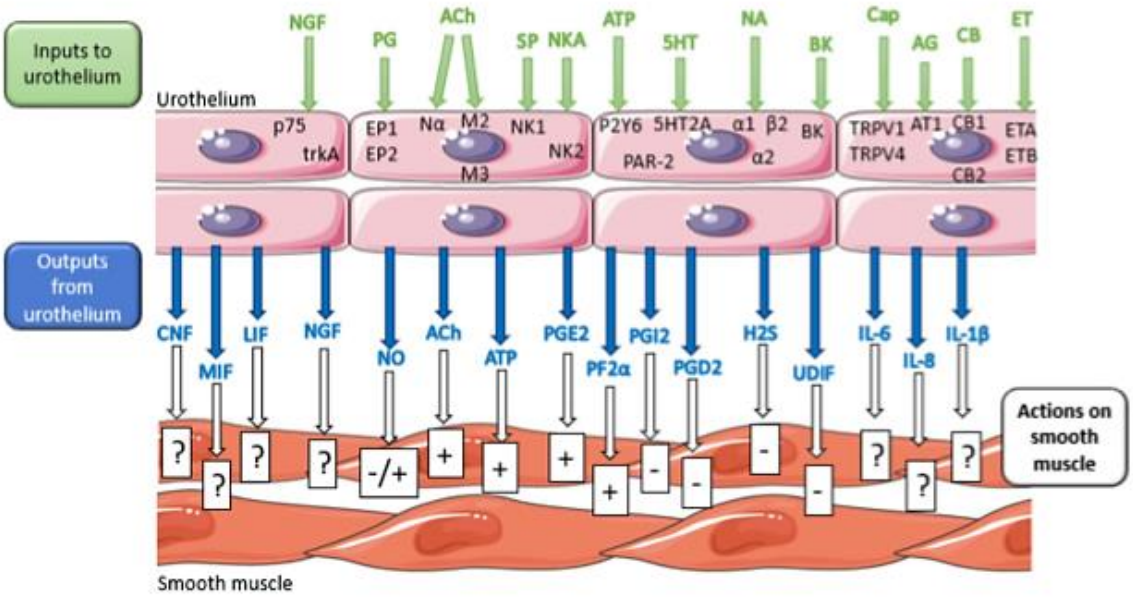


Figure 1.7: The many receptors found in the urothelium that can respond to a variety of inputs and the mediators that can be synthesized and released by the urothelium. The effects of urothelially released mediators on the bladder smooth muscle are (+) excitatory, (-) inhibitory and (?) unknown (reproduced with permission from the publisher, (Sellers et al., 2018)).

Urothelial ATP and purinergic receptors

The majority of ATP found in the bladder is believed to originate from the urothelium/lamina propria (Kumar et al., 2004). Mechanical stretch of isolated urothelium/lamina propria strips and cellular stretch of cultured urothelial cells and urothelial cell lines by osmotic swelling results in its release in many species including pig and human (Kumar et al., 2004, Sun and Chai, 2006, Cheng et al., 2011b, Smith et al., 2014, Sui et al., 2014, Farr et al., 2017). The release of ATP also occurs into the rodent bladder lumen on distension when distended which advocates that bladder filling triggers non-neuronal ATP release (Daly et al., 2014, Grundy et al., 2018b). Urothelially released ATP is believed to be the primary source of excitation for bladder afferents and is an important regulator of bladder functioning (Ferguson et al., 1997, Kumar et al., 2007, Aizawa et al., 2011a). As various cells located within or near the urothelium express P2X and P2Y receptors, the urothelial release of ATP may also play a role in sensory functions such as mediating nociception (Burnstock, 2000, Cockayne et al., 2000, Vlaskovska et al., 2001, O'Reilly et al., 2002, Birder, 2004).

Studies on the rabbit urothelium/lamina propria have revealed that ATP is released from both the luminal and basolateral surfaces (Wang et al., 2005, Lewis and Lewis, 2006). It has been proposed that ATP release from the basal surface if the urothelium in response to stretch acts on the P2X receptors on the sub-urothelial afferents depolarizing them, thus initiating the afferent pathway involved in the micturition reflex. Simultaneously, ATP release from the luminal surface of the urothelium activates the purinergic receptors on the urothelium which can further alter its own release and the release of other mediators (Cockayne et al., 2000, Vlaskovska et al., 2001, Chopra et al., 2008, Ferguson et al., 2015, Dunton et al., 2018).

Urothelial adenosine

After its release, ATP is rapidly converted to adenosine that acts on adenosine receptors A_1, A_{2a}, A_{2b} and A_3 (Schulte and Fredholm, 2003, Yu et al., 2006). The A1 and A3 receptors couple to G_i proteins thereby inactivating adenylate cyclase and reducing cAMP levels whereas A2a and A2b receptors couple with G_s proteins, activating adenylate cyclase increasing the production of cAMP (Ralevic and Burnstock, 1998).

Yu et al. (2006) found that hydrostatic stretch of the rabbit urothelium/lamina propria produced adenosine from the luminal and basolateral surfaces. However, the basolateral surface produced significantly more which is not surprising since the enzymes that degrade ATP to adenosine have been found more abundantly in the basal region of the urothelium (Yu, 2015). Immunolabelling has identified all four adenosine receptors in the urothelium, and overall it was found that A_1 receptors predominated the apical surface of umbrella cells surface while the A_{2a} receptors were found in all three urothelial layers but had a larger population just the below the surface of the urothelium (Yu et al., 2006). The A_{2b} and A_3 receptors were also found in all the urothelial layers with evidence of the A_{2b} receptor present in the detrusor. Although the function of adenosine and its receptors in the urothelium/lamina propria is not fully understood, activation of predominantly A_1 receptors and to a lesser extent A_{2a} receptors was found to contribute to the increased surface area of the umbrella cells. Therefore, urothelial adenosine receptors may contribute to the modulation of the apical surface when the bladder is filling (Yu et al., 2006). Additionally, Dunning-Davies et al. (2013) found that the activation of the A_1 receptor on the rabbit bladder by adenosine reduced stretch-induced urothelial ATP release and indicated that the reduction might be due to decreased levels of intracellular Ca^{2+}.

Urothelial P2Y receptor

There is limited information regarding P2Y distribution and function in the human urothelium. However, mRNA transcripts for P2Y1, P2Y2 and P2Y4 have been detected in freshly isolated samples of the urothelium, cultured urothelial cells and UROtsa immortalized human urothelial cell lines. (Save and Persson, 2010, Shabir et al., 2013). Immunofluorescence of the P2Y 1, 2 and 4 receptors have been identified in the cat urothelium, P2Y2 in the rat urothelium and P2Y6 on the guinea pig urothelium (Birder et al., 2004, Sui et al., 2006, Chopra et al., 2008). More recently, immunofluorescence of the P2Y6 receptor has been identified throughout the human urothelium and in the sub-urothelial layer (Silva et al., 2015).

The function of urothelial P2Y receptors is unclear at present. However, its primary role may be to amplify urothelial ATP. Studies have shown that activation of the P2Y receptors on rat cultured urothelial cells, human and guinea pig urothelium/lamina propria preparations evokes the release of ATP (Chopra et al., 2008, Sui et al., 2014, Silva et al., 2015) Furthermore, it has been demonstrated that the elevations in ATP release found in obstructed bladders were diminished by a P2Y6 antagonist (Silva et al., 2015). Additional roles for the P2Y receptor may include contributions to bladder capacity during distension as Wang et al. (2005) reported that P2Y agonists produced increases in the surface area of umbrella cells in rabbit urothelium/lamina propria. Much evidence is also accumulating that urothelial P2Y receptors contribute to immunity including phagocytosis, chemotaxis and cytokine production. It has been reported that P2Y receptors on kidney A498 and urothelial UROtsa cell line produced a concentration-dependent pro-inflammatory interleukin 8 (IL-8) release when exposed to the stable ATP analog ATP-γ-S (Save and Persson, 2010).

Urothelial P2X receptor

Immunoreactivity for the P2X2,3,5, and 7 receptors has been observed in the human urothelium along with mRNA expression of the P2X4 receptor (O'Reilly et al., 2002, Shabir et al., 2013, Svennersten et al., 2015). Although the exact role of the P2X receptors in the urothelium is not fully understood, P2X3 receptors are generally associated with sensory innervation and have been associated with the sensation of pain. Studies have suggested that both urothelial P2X2 and P2X3 receptors may play a significant role in transmitting sensations of bladder fullness and pain to the central nervous system as P2X3 KO were found to be less sensitive to pain, less frequent voiders and had a greater bladder capacity (Ferguson et al., 1997, Vlaskovska et al.,

2001, Tempest et al., 2004). Pharmacological inhibition of the urothelial P2X3 in rat bladders altered *in vivo* cystometry measurements which resulted in increased inter-contractile intervals (ICI) and bladder capacity and decreases to bladder peak pressure which has some similarities to the earlier KO models. Interestingly, blockade of the urothelial P2X3 receptors also modified detrusor contractions to purinergic stimulation (Ferguson et al., 2015).

The P2X7 receptor is activated at high concentrations of ATP and has been implicated in the inflammatory process (North, 2002, Donnelly-Roberts et al., 2008). Furthermore, In hTERT-immortalized human urothelial cells, the P2X7 receptor has been shown to participate in ATP release (Negoro et al., 2014). Cultured rat urothelial cells exposed to elevated and storage hydrostatic pressures that mirrored those of bladder outlet obstruction identified significant increases to ATP in the media along with intracellular caspase-1 activation. Antagonizing the P2X7 receptors resulted in reductions to pressure induced ATP and caspase-1 activation while antagonizing the P2X4 receptors resulted in decreases to caspase-1 activation and suggests that both receptors are involved in the production of ATP-mediated inflammatory cytokines in bladder pathology (Dunton et al., 2018). The P2X5 receptor, located specifically on normal human urothelial tissue (in very low levels) has been reported to be unlikely to have any significant contribution to bladder function (O'Reilly et al., 2002).

Urothelial ACh and cholinergic receptors

Like urothelially released ATP, mechanical stretch evokes the release of non-neuronal ACh from isolated urothelium/lamina propria strips in humans and pigs and other animals (Yoshida et al., 2006, Smith et al., 2014, Nile and Gillespie, 2012). The urothelial release of ACh is also induced in by hypertonic stimulation of cultured cells and human urothelial cell lines and into the lumen of the rodent bladder when distended (Hanna-Mitchell et al., 2007, Daly et al., 2014, Farr et al., 2017, Grundy et al., 2018b). Although the role of non-neuronal ACh in the urothelial signaling is not fully understood yet, it would most likely interact with muscarinic and nicotinic receptors on the urothelium and on the structures below such as afferent nerves and interstitial cells (Bschleipfer et al., 2007, Grol et al., 2009, Nandigama et al., 2010, Nandigama et al., 2013). Moreover, a substantial portion is thought to originate from the urothelium as stretch-induced ACh release declines considerably following the removal of the urothelial layer in the human bladder (Yoshida et al., 2006).

From what is understood of the non-neuronal ACh signalling pathway in the rat bladder, the instillation of ATP and an ACh analogue into the lumen cause contraction. The contraction caused by ATP is attenuated by the removal of the urothelium and by atropine (muscarinic antagonist) suggesting that urothelial ATP acts indirectly by promoting the release of ACh that promotes contraction as the removal of urothelium did not alter the contraction response to ACh analog (Stenqvist et al., 2017). This is supported by earlier experiments that found ATP promoted the release of ACh in cultured rat urothelial cells and strips of guinea pig urothelium/lamina propria (Hanna-Mitchell et al., 2007, Nile and Gillespie, 2012). However, it has also been demonstrated that muscarinic stimulation elicits ATP release in cultured rat and guinea pig urothelial cells, human and guinea pig strips of urothelium/lamina propria (Kullmann et al., 2008b, McLatchie et al., 2014, Sui et al., 2014). Furthermore, ACh may act in an auto feedback manner suppressing its own release as increases in ACh release to exogenous ACh were observed in the presence of atropine (muscarinic antagonist) in cultured rat urothelial cells, which highlights the complexities surrounding urothelial ACh signalling (Hanna-Mitchell et al., 2007).

Urothelial muscarinic receptors

Bschleipfer et al. (2007) has identified that the human urothelium expresses mRNA for all five muscarinic receptor subtypes (M1-M5). Follow up immunohistochemistry regarding their distribution throughout the urothelium has revealed that that M1 was restricted to the basal layer, M2 on the umbrella cells and M3,4 and 5 were distributed throughout the layers. These findings are supported by earlier immunohistochemistry and binding studies (Mansfield et al., 2005, Mukerji et al., 2006b) Using a variety of techniques, many studies have identified a larger population of urothelial M2 receptors followed by the M3 receptors (Mansfield et al., 2005, Bschleipfer et al., 2007, Braverman et al., 2007, Ochodnicky et al., 2012). The density of muscarinic receptors in the bladder appears to be species specific. In the human bladder, there appears to be a greater density of muscarinic receptors in the detrusor compared to the urothelium whereas in the pig the urothelium has a greater density compared to the detrusor (Hawthorn et al., 2000, Mansfield et al., 2005, Braverman et al., 2007).

Urothelial muscarinic stimulation has been found to enhance intracellular Ca^{2+} in cultured rat urothelial cells while in the same animal *in vivo* it has been implicated in regulating bladder contractility (inhibitory and excitatory) by multiple mechanisms involving urothelially released

mediators such as NO and ATP that can, in turn, modulate afferent nerve activity (Kullmann et al., 2008b, Kullmann et al., 2008a). Urothelial muscarinic receptors may also play a role in regulating spontaneous activity in this tissue (Moro et al., 2011). Interestingly, urothelial muscarinic receptors have also been shown to participate in urothelial proliferation and lipopolysaccharide-induced apoptosis and cellular proliferation (Arrighi et al., 2011, Podmolikova et al., 2018). This suggests their additional role in urothelial remodelling that occurs during pathophysiological conditions.

Urothelial nicotinic receptors

There are currently seventeen different subunits of nicotinic receptors that have been identified and these form pentameric channels that have been classified as α 1-10, β 1-4, γ, δ, and ε. They are divided into neuronal nicotinic receptors (α 2-10, β 2-4) and muscle nicotinic receptors (α 1, β 1, γ, δ, and ε). Neuronal nicotinic receptors can be further subdivided into three more groups which are; homomeric receptors such as α7 or α9, simple heteromeric receptors that contain and α and a β subunit and complex heteromeric receptors which contain three or more different subunits. Each type of receptor has different electrophysiological and pharmacological characteristics allowing for the varying effects of ACh (Beckel et al., 2006).

Nicotinic receptors have been found on the urothelium of rodents and humans. In humans, mRNA expression and immunohistochemistry have identified α7, α9 and α10 receptors. The α9 receptors were found in the superficial cells while the α7 and α10 receptors were present in all layers of the urothelium (Bschleipfer et al., 2007, Zarghooni et al., 2007, Bschleipfer et al., 2012, Beckel and Birder, 2012).

Stimulation of the nicotinic receptors on cultured rat urothelial cells increases intracellular Ca^{2+} and enhances or inhibits ATP release depending on subtype stimulation (Beckel et al., 2006, Beckel and Birder, 2012). The α3 receptor has dual stimulatory and inhibitory effects in rat bladders depending on the concentration of agonist. Stimulation of the α3 receptors with agonist at low concentrations decreases basal ATP release whereas higher concentrations enhance ATP release and decrease the ICI. The stimulatory effects were abolished by blocking the ATP response (Beckel et al., 2006, Beckel and Birder, 2012).

On the other hand, stimulation of the α7 receptors has a predominantly inhibitory effect. In cultured rat urothelial cells and urothelial tissue, its activation with choline inhibited basal ATP

release while *in vivo* choline increased the ICI (Beckel et al., 2006, Beckel and Birder, 2012). This is interesting when you consider that choline is a by-product of ACh degradation and in the human urothelium, the most abundant nicotinic receptor mRNA transcript was the α7 receptor (Bschleipfer et al., 2007). Similar to the muscarinic receptors, the nicotinic receptors in the urothelium can modulate bladder activity by complex mechanisms involving the release of mediators and have been shown to modify afferent activity when stimulated (Yu et al., 2016).

Nicotinic receptors may also play a part in regulating bladder tonicity as mice lacking α 3, β2 and β4 receptors displayed an absence of bladder contractility in response to nicotine, severe bladder distension, dribbling urination, infection and urinary stones (Xu et al., 1999a, Xu et al., 1999b).

Urothelial nitric oxide

The gaseous transmitter, nitric oxide (NO) can come from multiple sources in the bladder including the urothelium/lamina propria (Munoz et al., 2010, Mossa et al., 2018). Immunohistochemistry has identified eNOS and nNOS in the human urothelium/lamina propria while iNOS has been found in pathological conditions (Smet et al., 1996, De Ridder et al., 1999, Fathian-Sabet et al., 2001, Logadottir et al., 2013). In the guinea pig bladder body, the basal cells in the urothelium do not have elevated levels of cGMP in the presence of a NO donor. However, they are strongly immunoreactive to nNOS that suggest their primary role is to produce NO (Gillespie et al., 2005). The rodent urothelium releases NO in response to stimulation of the muscarinic, β-adrenergic and TRPV1 receptors while SP elicits NO release from the lamina propria (Birder et al., 1998, Munoz et al., 2010, Mossa et al., 2018, Birder et al., 2001)

The smooth muscle in the human urethra and the interstitial cells are highly immunoreactive to the NO induction of cGMP, the urothelium in the human bladder body also has a strong immunoreaction to cGMP and target enzyme soluble guanylyl cyclase compared to the detrusor suggesting that the urothelium is also a target for NO (Fathian-Sabet et al., 2001, Smet et al., 1996). Nitric oxide is associated with inhibitory activity, and while it seems not to have much direct influence on the detrusor, its actions in the urothelium/lamina propria may act indirectly as it has been shown to modulate spontaneous activity, afferent impulses and the release of excitatory transmitters (Aizawa et al., 2011b, Nile and Gillespie, 2012, Moro et al., 2012). Urothelial NO may also modify intravesical bladder pressure in response to the storage and

filling phase of the micturition cycle as β-adrenergic stimulation has been shown to elicit its release (Birder et al., 1998, Mossa et al., 2018).

Urothelial TRPV1 receptors

In humans and rodents, TRPV1 channel expression has been identified on the urothelium. In the human urothelium, TRPV1 immunoreactivity was observed in the basal and apical cell layers. In whole bladder preparations, the mRNA expression of the TRPV1 channel was higher in the urothelium when compared to the detrusor (Lazzeri et al., 2004b, Apostolidis et al., 2005, Liu and Kuo, 2007a, Liu et al., 2007).

Like other TRPV1 channels found in the bladder, urothelial TRPV1 is suspected to be involved in sensory transmission responding to its chemical and physical environment. Urothelial mRNA expression of the TRPV1 channel was found to be up-regulated and inversely correlated with the first desire to void in patients with sensory urgency (Liu et al., 2007). Moreover, it has been shown that activation of the TRPV1 channel with capsaicin and resiniferatoxin in the rodent urothelium induces NO release while TRPV1 KO mice produce significantly reduced levels of stretch evoked ATP release (Birder et al., 2001, Birder et al., 2002, Grundy et al., 2018b). On the other hand, in rats, the consequence of subcutaneous phenylephrine and subsequent α1-adrenoceptor stimulation produced bladder pain and increased voiding contractions compared to control animals. In the same experiment, it was found in cultured human urothelial cells that α1-adrenoceptor stimulation with phenylephrine enhanced ATP release that was attenuated by TRPV1 channel antagonist which lends further support to its influence in sensory pathways in the urothelium (Matos et al., 2016).

Urothelial Nerve Growth Factor

The role of nerve growth factor (NGF) in the bladder generally participates in neuron development and maintenance of sympathetic and afferent fibres including regulating expression levels of afferent transmitters such as SP and CGRP (Lindsay and Harmar, 1989, Steers and Tuttle, 2006). Nerve growth factor binds to tropomyosin-related kinase receptor-A (trkA) and p75 receptors. It is generally thought that the trkA receptor mediates growth and survival whereas the p75 receptor promotes cellular apoptosis. However, the interaction that NGF has with these receptors is much more complex as they can be individually or co-expressed modifying the effects of NGF (Lad et al., 2003, Kalb, 2005).

In humans and rodents, mRNA expression and immunoreactivity of NGF and its receptors (trkA and p75) have been identified by throughout the bladder including the urothelium (Shibayama and Koizumi, 1996, Vaidyanathan et al., 1998, Teng et al., 2002, Liu et al., 2010, Girard et al., 2011, Giannantoni et al., 2013). NGF interacting with trkA receptors on cultured human urothelial cells has been shown to participate in cellular proliferation (Teng et al., 2002). Although NGF is required for normal development and maintenance, it can be over-expressed in the urothelium/lamina propria in inflammatory conditions (Lowe et al., 1997, Coelho et al., 2015). Moreover, NGF delivered intravesically is associated with bladder overactivity and hypersensitivity of the Aδ and C-fibres in the rat (Dmitrieva and McMahon, 1996, Zvara and Vizzard, 2007). In cultured rat urothelial cells, NGF has been shown to enhance the expression of TRPV1 channels which was prevented by antagonizing the trkA receptors (Coelho et al., 2015). Although the TRPV1 channel has previously been associated with pain and overactivity in the bladder but not in the induction of inflammation, in the previously mentioned study, antagonizing the trkA receptors *in vivo* prevented activation of the pain signalling pathway in response to cyclophosphamide-induced inflammation which demonstrates the contribution of the TRPV1 channel in pain sensation in the bladder. (Charrua et al., 2007, Mingin et al., 2015, Coelho et al., 2015).

Urothelial prostanoids

Like ATP, ACh and NO, prostanoids are additionally released from the urothelium in response to stretch and distension (Jeremy et al., 1987, Tanaka et al., 2011, Farr et al., 2017). In many studies, the majority of prostaglandins have been found in the urothelium when compared to the detrusor (Masick et al., 2001, Azadzoi et al., 2004, Guan et al., 2014).

The administration of PGE_2 into human and rodent bladders *in vivo* has a facilitatory effect on the micturition reflex (Schüßler, 1990, Takeda et al., 2002, Wang et al., 2008). The administration of PGE_2 on cultured mouse urothelial cells has elicited the release of ATP while intravesical administration into rat bladders activates C-fibres (Wang et al., 2008, Aizawa et al., 2010b). Moreover, its release can be modulated by other urothelial transmitters such as ATP, ACh and NO (Nile et al., 2010, Nile and Gillespie, 2012). With its documented facilitatory effects in the bladder, PGE_2 has been suspected to be a contributing factor in patients with overactive bladders (Kim et al., 2006, Cho et al., 2013).

Urothelially derived inhibitory factor

It was observed in bladder strips from the pig that the removal of the urothelium/lamina propria elicited a much greater contractile response from the remaining detrusor tissue in response to muscarinic agonists. The inhibitory effect of the urothelium remained unchanged by inhibiting COX, NO, purinergic, adrenergic and K^+ pathways. The mechanism behind this response involves the release of a diffusible factor termed urothelially derived inhibitory factor (UDIF) which is yet to be identified, however, NO, prostanoids, adenosine nucleotides, catecholamine's, gamma-aminobutyric acid and apamin-sensitive response to endothelium-derived hyperpolarizing factor have been ruled out (Hawthorn et al., 2000). The inhibitory factor has also been identified in other species such as the mouse and human bladder (Canda et al., 2009, Propping et al., 2013). In human bladder tissue, the response to muscarinic agonist, parasympathetic stimulation by electrical field stimulation (EFS) and potassium chloride (KCl) but not NKA is enhanced minus the urothelium/lamina propria (Chaiyaprasithi et al., 2003, Propping et al., 2013). The presence of the urothelium/lamina propria in the human bladder has also been found to inhibit the relaxation response to noradrenaline that involves urothelial β2-adrenoceptors. It is uncertain whether UDIF is a product of the urothelium or lamina propria, as removal of the urothelial cell layer in the rat bladder by swabbing rather than surgical removal of the urothelium/lamina propria produced depressed responses to EFS, muscarinic and purinergic stimulation suggesting the lamina propria is the site of UDIF production (Munoz et al., 2010).

Sub-urothelial interstitial cells

Numerous receptors and ion channels that are normally associated with sensory neurons have also been identified on the network of sub-urothelial interstitial cells, which include purinergic receptors, muscarinic receptors, prostaglandin EP1 and EP2 receptors and TRPV channels. (Sui et al., 2006, Mukerji et al., 2006b, Birder et al., 2010b, Andersson and McCloskey, 2014, Heppner et al., 2017). Moreover, due to their close approximation to the urothelium, nerves and their connectivity with gap junctions suggest their role in transferring or propagating signals from the urothelium throughout the bladder (Wiseman et al., 2003, Kanai et al., 2007, Rahnama'i et al., 2012a, Neuhaus et al., 2018).

In Support of their hypothesized role and extending on earlier reports on the guinea pig and human sub-urothelial interstitial cells in regards to ATP inducing and intracellular Ca^{2+}

response, Heppner et al. (2017) found that exogenous ATP on interstitial cells in the lamina propria of rat pups increased Ca^{2+} transients that culminated into numerous Ca^{2+} waves that propagated throughout the lamina propria. (Wu et al., 2004, Sui et al., 2008, Cheng et al., 2011a, Heppner et al., 2017). These findings are similar to an earlier report by Kanai et al. (2007) who demonstrated signal transfer between the urothelium/sub-urothelium and detrusor in the rat pup bladder by measuring the propagation of intracellular Ca^{2+} and subsequent membrane potential waves. In response to muscarinic stimulation and stretch, the waves originated in the urothelium/sub-urothelium and after a short delay, propagated towards the detrusor. In the adult rat bladder under the same conditions, the spread of Ca^{2+} and membrane potential waves throughout the urothelium/sub-urothelium regions and did not propagate to the detrusor which may allow for conscious voiding in the mature adult (Kanai et al., 2007).

The evidence clearly shows that the urothelium in addition to its role as a barrier is a sensory tissue that interacts with its environment to communicate with the CNS via afferent innervation and interstitial cells found in the lamina propria. The urothelium/lamina propria also releases mediators which can modulate overall bladder activity.

Many of the receptors mentioned above and signalling mediators that are expressed throughout the bladder, particularly from the urothelium can be altered or dysfunctional in diseased states such as interstitial cystitis/bladder pain syndrome.

1.5 Interstitial cystitis/bladder pain syndrome

Overview

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a condition characterized by pelvic pain and urinary storage symptoms such as urgency, frequency and nocturia in the absence of bacterial infection or other identifiable pathology (Dell et al., 2009, Daniels et al., 2018). Bladder pain while producing small amounts of urine is typical of this disease. It is relieved by voiding but soon reoccurs while the bladder is filling. The constant urge to void is exceptionally uncomfortable with urination frequency of up to 40 times within a 24-hour period (Erickson and Davies, 1998). Bladder cytometric data from healthy middle-aged women shows a mean bladder capacity of 586 mL and a first desire to void volume of 163 mL, on the other hand, patients with IC/BPS have substantially lower cytometric bladder volumes, with a mean bladder capacity of 265 mL and a first desire to void volume of only 74 mL (Steinkohl and

Leach, 1989, Pauwels et al., 2004). It has been recently identified in patients with IC/BPS that a smaller bladder capacity is associated with more intense symptomology (Mazeaud et al., 2018). Symptoms can be exacerbated by stress and certain foods such as coffee, chocolate, carbonated drinks, alcohol, citrus and tomatoes. In women, dyspareunia may occur or result in a flare-up the next day and symptoms are often worse the week before menses. The overall course of this disease is marked by flare-ups and remissions (Erickson and Davies, 1998, Metts, 2001, Daniels et al., 2018).

A brief history of IC/BPS

Nitze first described IC/BPS in the German medical literature in 1907 and it became popularized by Guy Hunner in 1915. Hunner (1915) cystoscopically identified bladder ulcers (Later coined as "Hunner's ulcer") and bladder inflammation, particularly in the urethra in women presenting with the symptoms mentioned above (Hunner, 1915, Bohne and Fetz, 1954). It is important to note that "Hunner's ulcer" may not be apparent in the initial stages of this disease. In fact, Messing and Stamey (1978) felt that relying on the presentation of an ulcer has done more in preventing the recognition of IC/BPS than any other factor. Bladder pain syndrome patients can be divided into two categories such as those presenting with "Hunner's" ulcer and those who present without ulceration. If left untreated, IC/BPS can produce progressive fibrosis of the bladder musculature, severely diminished bladder capacity and total loss of function (Martin, 1963, Jacobo et al., 1974).

Establishing a diagnosis

A diagnosis of IC/BPS is usually tricky and delayed, as symptoms can overlap with other urological and gynaecological conditions (Cervigni and Natale, 2014). On average, it has been reported that patients visited two medical institutions and needed more than three years to obtain a diagnosis (Ito et al., 2007). Differential diagnosis may include urinary tract infection, endometriosis, chronic pelvic pain and vulvodynia. For a more comprehensive list see **Table 1.1** (Dell et al., 2009, Cervigni and Natale, 2014).

Diagnosis of IC/BPS is by exclusion and the diagnostic criteria set out the American Urological Association (AUA) and European Association of Urology (EAU) recommended that all confusable diseases with overlapping symptoms relatable to IC/BPS be ruled out before a diagnosis can be made (Hanno et al., 2011a, Hanno et al., 2015, Engeler et al., 2018). After

confusable diseases have been ruled out, the current EAU guidelines have listed patients could then be diagnosed based on; chronic pelvic pain (>3 months), pressure and discomfort related to the bladder and at least one other accompanying urinary symptom such as voiding frequency or persistent urge to void (Engeler et al., 2018). However, more inclusive criteria put forward by the AUA in 2014 recommends pain and symptoms of more than six weeks in duration to improve diagnosis and treatment outcomes (Hanno et al., 2015).

Cystoscopy with hydrodistension and a biopsy may also be required to make a definite diagnosis. However, the AUA guidelines state it should be considered whereas the EAU recommends it. Cystoscopic features of IC/BPS are the presence of strawberry-like dots called glomerulations, Hunner's ulcer or both features. Positive biopsy findings are inflammatory infiltrates, granulation tissue, detrusor mastocytosis and intrafascicular fibrosis (van de Merwe et al., 2008, Hanno et al., 2011b, Engeler et al., 2018).

The cystoscopic findings can be linked with epidemiological evidence such as the age of onset, relief of pain on standing, the location of pain and the presence of blood in urine. However, the epidemiological evidence alone cannot distinguish between ulcerated and non-ulcerated forms of the disease with a high degree of accuracy (Koziol et al., 1996). Also, cystoscopic observations by Messing and Stamey (1978) and Tomaszewski et al. (2001) found that the presence or severity of glomerulations or the presence of Hunner's ulcer in patients with IC/BPS was not predictive of primary symptoms.

Interstitial cystitis/bladder pain syndrome is typically diagnosed in midlife, although children can be affected. Paediatric prevalence is unknown as patients under 18 years of age are excluded in criteria determined by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (Hanno et al., 1999). Interestingly, in Hunner's original findings of 1915, he determined that the average age of onset for his IC/BPS patients was twenty years of age or earlier (Hunner, 1915).

The incidence of IC/BPS

It is estimated that approximately >2% of females are affected by IC/BPS (Konkle et al., 2012). For female patients with IC/BPS, there is usually a family history and a higher occurrence of recurrent urinary tract infections and antibiotic use in childhood (Peters et al., 2009). Historically, the female to male ratio is 10:1, however, it has been suspected that the incidence

of male IC/BPS may be higher than previously thought. Although being a woman is a risk factor it has been suggested that it is underdiagnosed especially in males (Forrest and Vo, 2001, Davis et al., 2015). Furthermore, it has been observed that some men with chronic non-bacterial prostatitis may have IC/BPS based on hydrodistension and cystoscopic findings (Berger et al., 1998). This is interesting considering that cystoscopy is less commonly performed in men with the same symptoms reported by women (Payne et al., 2007).

There are consistent reports of the association of IC/BPS with other chronic pain conditions, such as fibromyalgia, chronic fatigue syndrome and irritable bowel syndrome (McLennan, 2014, Van Moh et al., 2018). Mental health issues, particularly the incidence of depression and panic disorder has also been reported to be significantly higher in IC/BPS patients (Clemens et al., 2008). A recent report identified that anxiety attacks are reported more often in IC/BPS patients that present without Hunner's lesions (Van Moh et al., 2018).

The impact of IC/BPS

The chronic and recurring symptoms of IC/BPS can be debilitating, negatively impacting the quality of life for patients. Compared to control patients, sufferers of IC/BPS have reported significant increases in sleep dysfunction, depression, catastrophizing, stress and anxiety with moderately increased social and sexual dysfunction (Nickel et al., 2010).

The economic impact of IC/BPS is substantial and is likely underestimated due to delays in diagnosis or misdiagnosis. The economic impact is both direct and indirect. The *direct* impact depends on the country, the level of health care provision and includes payments such as payments to physicians for in and outpatient care, payments to the hospital for inpatient care, payments for outpatient procedures, tests and the cost for prescriptions. It has been reported that the *direct* financial impact of the disease on the individual is generally greater than other chronic pain conditions and two-fold more compared to individuals without the disorder (Payne et al., 2007, Clemens et al., 2009). As patients are typically affected during their most productive years for work and family life, the *indirect* impact involves lost income due to time spent away from work. The employer bears costs due to sick leave and loss of productivity. Indirect costs are difficult to measure and include un-pursued work, educational and social opportunities, low quality of life, loss of social and family support, depression, divorce and in some cases suicide (Payne et al., 2007, Clemens et al., 2009, Beckett et al., 2014).

Table 1.1: Differential diagnosis for IC/BPS including confusable diseases that are required to be ruled out before making a positive diagnosis (van de Merwe et al., 2008, Cox et al., 2016).

IC/BPS Differential Diagnosis	Excluded By:
Carcinoma / Carcinoma in situ	Cystoscopy and biopsy
Infection: ***Chlamydia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis, Mycoplasma genitalium, Corynebacterium urealyticum*** **and Candida species.** ***Mycobacterium tuberculosis***	Routine bacterial culture/special cultures Dipstick, if sterile pyuria culture for *M. tuberculosis*
Human papilloma virus and Herpes simplex	Physical examination
Chemotherapy including immunotherapy with cyclophosphamide	Medical history
Radiation	Medical history
Bladder neck obstruction and neurogenic outlet obstruction	Ultrasound and uroflowmetry
Anti-inflammatory therapy with tiaprofenic acid	Medical history
Lower ureteric stones	Medical history/ hematuria in the upper urinary tract. Imaging: CT or IVP
Bladder stones	Imaging/ cystoscopy
Vaginal candidiasis	Medical history/ physical exam
Endometriosis	Medical history/ physical exam
Urethral diverticulum	Medical history/ physical exam
Urogenital prolapse	Medical history/ physical exam
Cervical, ovarian & uterine cancer	Physical exam
Overactive bladder	Medical history/ urodynamics
Incomplete bladder emptying	Post-void residual urine volume by ultrasound

Continued….

IC/BPS Differential Diagnosis	Excluded By:
Prostate cancer	Physical exam/ PSA
Chronic bacterial and non-bacterial prostatitis	Medical history/ physical exam/ culture
Benign prostatic obstruction	Uroflowmetry/ pressure flow studies
Pudendal nerve entrapment	Medical history/ physical exam (nerve block may prove diagnosis)
Pelvic floor muscle related pain	Medical history/ physical exam
Vulvar disorders	Medical history/ physical exam

Pathophysiology of IC/BPS

Multiple theories of its pathogenesis have been proposed over the decades however none fully explain the manifestations of the disease; therefore, its exact aetiology remains unknown. Moreover, the ulcerative and non-ulcerative presentations of IC/BPS may represent two separate conditions (Koziol et al., 1996, Davis et al., 2014). As the underlying pathophysiology of IC/BPS is poorly understood, several theories persist although there is no consensus on classifying this condition. The possible pathological mechanisms with a focus on human studies will now be discussed.

Urothelial dysfunction

Some authors have described IC/BPS as a disease of the urothelium since one of the critical histological findings in patients with IC/BPS is thinning or complete erosion of the urothelium (Slobodov et al., 2004, Leiby et al., 2007, Birder, 2014, Lee and Lee, 2014). As one of the functions of the urothelium is to act as a barrier from urine and its solutes, it has been hypothesized that the consequence of a compromised urothelial barrier allows noxious solutes, in particular, potassium (K^+) to diffuse through the urothelium to the underlying structures. The solutes can depolarize the bladder nerves, initiate the cascade of inflammation, cause tissue injury and possibly cause an autoimmune reaction in bladder tissue (Moskowitz et al., 1994,

Parsons et al., 1998, Parsons, 2003, Parsons, 2007).

Supporting the concept of aberrant urothelial permeability, Buffington and Woodworth (1997) found that after oral ingestion fluorescein, which is used to measure membrane permeability, patients with IC/BPS had increased plasma concentration of dye and decreased dye excretion in their urine compared to their control subjects. While K^+ levels are relatively high in human urine, urine from IC/BPS patient was found to contain significantly less K^+ than normal patients (Parsons et al., 2005). Interstitial cystitis/bladder pain syndrome patients were also more likely to experience pain when 0.4 M of KCl was instilled into the bladder (Parsons et al., 1994). Also, IC/BPS patients quite often experience exacerbation of their symptoms after consuming foods that are acidic and high in K^+ such as citrus and tomatoes (Shorter et al., 2007).

Disrupted GAG layer and tight junctions

The GAG layer of the urothelium plays a significant role in regulating permeability to urinary solutes (Ruggieri et al., 1994). The reason why the urothelium may become permeable in IC/BPS is unknown, but alterations in the urothelial GAG layer have been described. In biopsies from patients with IC/BPS, Hurst et al. (1996) found statistically significant decreases of chondroitin sulphate, a component of the GAG layer, on the luminal surface of the urothelium and in the lamina propria of patients with severe IC/BPS. In contrast, another study found that IC/BPS samples had a morphologically normal appearing GAG layer (Dixon et al., 1986). Similarly, other studies have also identified abnormalities in chondroitin sulphate and proteoglycans in IC/BPS patients compared to normal controls. However, a small number of these patients had no abnormalities. (Slobodov et al., 2004, Hauser et al., 2008).

Uronic acid, also a component of the GAG layer was measured in the urine of IC/BPS patients and was found to be reduced (Parsons and Hurst, 1990). In another study, uronic acid excretion was significantly elevated and associated with the more severe form of IC/BPS (Lokeshwar et al., 2005). In agreement with Lokeshwar et al. (2005), an earlier study by Wei et al. (2000) also found an elevation in total urinary GAGs in patients who had more moderate to severe symptoms.

Decreases to proteins such as zona occludens (ZO-1) and occludins that are responsible for urothelial tight junctions have been found in IC/BPS samples. (Slobodov et al., 2004, Lee and Lee, 2014, Ong and Kuo, 2017). Similar to components in the GAG layer, some IC/BPS

samples also had no abnormalities in ZO-1 in either presentation of IC/BPS (non-ulcerative vs. ulcerative) (Slobodov et al., 2004, Jhang et al., 2016). On the other hand, there are reports that the urothelial adhesion protein E-cadherin is reduced in non-ulcerative and ulcerative forms of IC/BPS (Shie and Kuo, 2011, Jhang et al., 2016, Ong and Kuo, 2017). In contrast, Slobodov et al. (2004) and Zhang et al. (2005) identified increased expression of E-cadherin in the urothelium of IC/BPS patients. Taken together, these findings suggest that disruption to the GAG layer and disruption to urothelial tight junctions and adhesion proteins is highly variable in all cases of IC/BPS and their alteration may be a progressive manifestation of the disease or different pathology. Interestingly, a negative correlation was found to exist between E-cadherin expression and pain scores (Shie and Kuo, 2011).

Altered receptors and mediator release

In addition to the structural defects of the urothelium and its barrier function associated with IC/BPS, there is also evidence of several urothelial mediator and receptor modifications. These include anti-proliferative factor, ATP and purinergic receptors, ACh and cholinergic receptors, NO, NGF and prostaglandins.

Anti-proliferative factor

Anti-proliferative factor (APF) is a low molecular weight glycosylated peptide released specifically from IC/BPS affected urothelial cells and has been named the likely culprit responsible for the thinning and denudation of the urothelium that is seen in IC/BPS patients (Keay et al., 2000). Anti-proliferative factor has been implicated in the profoundly lower proliferation rates of urothelial cells in IC/BPS patients (Keay et al., 2003b). Urothelial cells from IC/BPS samples and normal urothelial cells treated with APF were found to have altered gene expression that may involve down-regulation of genes that stimulate proliferation and up-regulation of genes that inhibit cell growth (Keay et al., 2003a).

In the urine of IC/BPS patients, urothelial cell growth factors such as heparin-binding epidermal growth like factor (HB-EGF), known to stimulate log-phase growth in the urothelium, were found to be significantly decreased in contrast to the increased urinary levels of epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1) and IGF binding protein 3 (Keay et al., 1997). Accordingly, when normal urothelial cells were exposed to APF, not only was HB-EGF production reduced by 80-90%, EGF, IGF1 and IGF binding protein 3,

production increased by 80-100% and paracellular permeability was enhanced (Keay et al., 2000, Zhang et al., 2005). Anti-proliferative factor has also been implicated the downregulation of ZO-1 and occludins that most likely increase the permeability of the urothelium (Zhang et al., 2005). Interestingly, an antagonist for APF was able to stimulate the expression of tight junction proteins including ZO-1 and occludin in cultured IC/BPS cells similar to normal expression in healthy cells (Keay et al., 2011). The presence of APF in urine has been suggested as a diagnostic test for IC/BPS due to its high sensitivity and specificity. Moreover, its levels were similar between ulcerative and nonulcerative forms of the disease (Kuo, 2014).

ATP and purinergic receptors

The urothelium/lamina propria from IC/BPS patients were found to have significantly elevated levels of ATP compared to control urothelium/lamina propria during basal resting conditions, in response to mechanical stretch and parasympathetic stimulation. These findings indicate there are increases to both non-neuronal and neuronal ATP as tetrodotoxin (TTX) reduced nerve-mediated ATP by about 75% which was similar to stable control urothelium/lamina propria (Kumar et al., 2007). An earlier report by Sun and Chai (2006) reported that urothelial cells affected by IC/BPS released significantly more ATP on exposure to increasing concentrations of exogenous ATP and proposed that a vicious cycle is occurring where the already increased levels of urothelial ATP may be promoting further ATP release which may produce the unpleasant symptoms. Some of the ATP enhancement found in IC/BPS affected cells was due to diminished ecto-nucleotidase activity. However, it did not account for the entire increase suggesting that other mechanisms are behind its elevation (Sun and Chai, 2006). Interestingly, in a follow-up study, it was found that in the presence of APF, healthy cultured human urothelial cells significantly enhanced ATP release induced by exogenous ATP demonstrating APF produces an IC/BPS phenotype in urothelial cells (Sun et al., 2009). The exact mechanism of how APF enhances ATP is uncertain and presents another mechanism behind the altered release.

Purinergic receptors also appear to be altered by this condition. Western blot analysis of urothelium/lamina propria from patients with IC/BPS identified an up-regulation of the P2X2 and P2X3 receptors, although an inverse correlation was determined in gene expression (Tempest et al., 2004). In contrast, more recent western blot analyses identified no changes to the P2X3 receptors in the urothelium/lamina propria between normal and IC/BPS samples

(Jhang et al., 2016, Ong and Kuo, 2017). It was found in earlier studies of cultured urothelial cells from IC/BPS patients that stretch conditions over a period of 96-hours resulted in a significant increase in the urothelial P2X3 receptor (Sun and Chai, 2004). And in regards to APF, when in the presence of normal cultured human urothelial cells, the introduction of exogenous ATP significantly increased P2X3 receptors after 3-hours as determined by fluorescence-activated cell sorting (FACS) analysis (Sun et al., 2009). These findings suggest that for the IC/BPS affected urothelium, there is a tendency for the P2X3 receptor to be upregulated in stimulated conditions and the contrasting findings found in western blot analysis of receptor population in the urothelium/lamina propria compared to healthy control urothelium/lamina propria may reflect the presence of lamina propria or tissue handling.

ACh and cholinergic receptors

In general, there is limited information regarding non-neuronal ACh in IC/BPS patients. However, in animal models, feline interstitial cystitis (FIC) closely resembles that of non-ulcerative IC/BPS in humans and meets all of the inclusion and assessable exclusion criteria as stated by the National Institutes of Arthritis, Diabetes, Digestive and Kidney diseases (Westropp and Buffington, 2002). Moreover, hydrodistension of cat bladders affected by FIC display enhanced permeability to water and urea. Morphologically there is urothelial thinning and denudation and a reduction in ZO-1 (Lavelle et al., 2000). Furthermore, alterations to urothelial purinergic receptors and enhanced ATP from cultured FIC urothelial cells to osmotic swelling has been described (Birder et al., 2003, Birder et al., 2004).

Ikeda et al. (2009b) found that in FIC affected cats, there were significantly more spontaneous Ca^{2+} signals that originated in the urothelium/sub-urothelium layer compared to normal cats. These signals evoked contractile activity that propagated to the detrusor and suggests that the urothelium/sub-urothelium in FIC may contribute to the enhanced sensitivity of this tissue. Moreover, the presence of a muscarinic agonist enhanced spontaneous activity and produced more Ca^{2+} signalling in the urothelium/sub-urothelium compared to normal cats which may reflect enhanced ACh release or altered muscarinic receptor expression. In support of enhanced ACh signalling in IC/BPS, carbachol (muscarinic agonist) on cultured urothelial cells from IC/BPS patients had enhanced potency and efficacy by increasing intracellular Ca^{2+} that was abolished by Ca^{2+} free conditions and tolterodine (muscarinic antagonist). The enhanced muscarinic signal to stimulation was suggested to be due to altered muscarinic receptor

expression, an alteration to the coupling of muscarinic receptors with the Ca^{2+} influx or alterations to the mechanisms that counteract Ca^{2+} influx (Gupta et al., 2009).

Immunohistochemistry studies on human bladders affected by IC/BPS identified no difference in urothelial expression of M2 and M3 receptors compared to the healthy bladder but rather identified increased expression of the M2 receptor on the interstitial cells in the sub-urothelium (Mukerji et al., 2006b). More recent western blot analyses found decreases in M3 receptor expression in urothelium/lamina propria samples from IC/BPS samples in both its ulcerative and non-ulcerative forms compared to healthy control tissues (Jhang et al., 2016, Ong and Kuo, 2017). As the lamina propria was included in the previously mentioned studies, the decreases in M3 receptor expression may come from other tissues in the lamina propria but not likely to be the interstitial cells as Mukerji et al. (2006b) could identify no change in M3 receptor immunoreactivity between IC/BPS and healthy samples.

Nitric oxide

Bladders from patients with IC/BPS produce significantly more luminal NO when compared to healthy control bladders (Lundberg et al., 1996, Ehren et al., 1999, Koskela et al., 2008). However, when Logadottir et al. (2004) compared non-ulcerating IC/BPS patients with an ulcerated form of the disease, it was found that luminal NO was very high in the ulcerated IC/BPS group whereas NO release in the bladder of patients with non-ulcerating IC/BPS was similar to control subjects. Thus, NO levels were not related to symptoms but rather the assignment of the disease. As the source of NO is from the lumen of the bladder in the previously mentioned studies, this indicates that the enhanced NO release is from the urothelium/lamina given the short half-life of NO (Renström Koskela and Wiklund, 2007). It is uncertain whether the elevated levels are damaging or protective, although when produced in excess, it can promote free radical formation, tissue damage, inflammation and may even promote the observed changes in urothelial integrity (Nathan and Xie, 1994, Koppenol et al., 1992).

Bladder specimens from patients with ulcerative and non-ulcerative IC/BPS reportedly express significantly higher levels of eNOS and iNOS. This expression of iNOS is confined to urothelial layer and is hypothesized to be responsible for the generation of increased NO observed in these patients (Ying and Hofseth, 2007, Koskela et al., 2008, Logadottir et al., 2013, Jhang et al., 2016).

Nerve growth factor

Immunostaining of NGF has identified its presence in the urothelial layers of patients affected by IC/BPS while none was detected in healthy control bladders (Lowe et al., 1997). Lowe et al. (1997) proposed that as the sensory afferent fibres terminate near urothelium, the elevated levels of NGF might act directly on the fibres producing enhanced bladder sensations and hyperalgesia experienced by these patients. Moreover, in bladders affected by IC/BPS increased severity of the condition correlated with increased levels of NGF (Liu et al., 2014). Also, in IC/BPS patients, urinary NGF levels were positively correlated with urgency with pain symptoms (Kim et al., 2014).

Overexpression of NGF is found in inflamed tissue and is ubiquitous with the inflammatory response (McMahon, 1996). The effects of urothelial NGF overexpression in the bladder have been demonstrated using an experimental mouse model. Nerve growth factor overexpression resulted in increased bladder weight due to nerve fibre hyperplasia, particularly in the sub-urothelium. Increases were found in Aδ -fibres, C-fibres and sympathetic fibres. There was also an increased population of mast cells throughout the bladder. Their location in the sub-urothelium was around blood vessels and nerves. The changes to bladder function included reduced bladder capacity, bladder hyperreflexia and referred somatic hypersensitivity (Schnegelsberg et al., 2010).

With regards to the receptors for NGF, immunohistochemistry studies from patients with ulcerating and non-ulcerating IC/BPS identified an up-regulation of the P75 receptor on basal urothelial cells (Regauer et al., 2017). In an abstract by Jhang et al. (2018a), western blot analysis identified a significant increase in urothelial TrkA expression in ulcerated IC/BPS compared to non-ulcerating IC/BPS and control samples. The upregulated expression of the TrkA receptors in the more severe form of IC/BPS correlated to clinical symptoms in which its expression may be related to increased levels of NGF.

Prostaglandins

Prostaglandin E_2 is associated with inflammation and pain, and its elevated presence has been identified in the urine of ulcerating IC/BPS patients compared to non-ulcerating IC/BPS and healthy control patients. Elevated levels were also found in the ulcerating form of IC/BPS in response to distention compared to the non-ulcerating type. Along with luminal elevations in

PGE_2 in this subset of patients, mRNA expression of EP1, EP2 and COX2 were also enhanced in the urothelium/lamina propria (Narumiya et al., 1999, Wada et al., 2015). Earlier studies on urinary PGE_2 in patients with IC/BPS identified no change compared to healthy control subjects. However, they did not discriminate between the ulcerating and non-ulcerating forms of this disease (Kim et al., 2014).

Altered nerve signalling.

Parasympathetic alterations

As mentioned earlier, atropine (muscarinic antagonist) resistant contractions to efferent nerve stimulation are not typically found in the healthy human bladder, but they do seem to play a role in bladder pathology. This was undoubtedly the case in detrusor strips taken from patients suffering from IC/BPS where the diseased strips all displayed a degree of atropine resistance in response to an electrically induced contraction that was identified to be ATP acting on the P2X receptors. No atropine resistance was found in the control samples from patients with stable bladders (Palea et al., 1993). Furthermore, in human detrusor biopsies from IC/BPS patients, immunohistochemistry results revealed a significant upregulation of P2X1 and P2X2 receptors which may be enhancing the contractile response in these bladders (Neuhaus et al., 2012).

Nerve-mediated ACh does not appear to be affected by IC/BPS as nerve-stimulated ACh release from FIC affected cats did not differ from healthy cats (Buffington et al., 2002). Although, Neuhaus et al. (2012) also identified a significant increase of the M2 receptor in the detrusor of IC/BPS patients. These findings indicate that the bladder has altered parasympathetic transmission in the detrusor by ATP interacting with elevated purinergic receptors while the consequence of ACh on elevated M2 receptors is unknown. However, in the neurogenic bladder, they have been shown to contribute to the contractile response (Pontari et al., 2004).

Sympathetic alterations

Interstitial cystitis/bladder pain syndrome is associated with increases in sympathetic nerve fibre expression in the detrusor and sub-urothelial layer (Hohenfellner et al., 1992, Peeker et al., 2000a) Supporting this finding is significantly higher levels of noradrenaline found in the urine of these patients (Charrua et al., 2015). Moreover, repeated exposure to phenylephrine

(α-adrenoceptor agonist) in the rat bladder induced increases to urinary frequency, urothelial thinning, increased urothelial permeability, increased mast cell infiltration in the sub-urothelial layer and increased pro-apoptotic proteins in the urothelium (Charrua et al., 2015). The excess release of noradrenaline producing the effects in the urothelium is likely to be secondary to enhanced levels of NGF which has been shown to promote sympathetic hyperinnervation in the bladder (Schnegelsberg et al., 2010). These findings also explain why symptoms in IC/BPS may be exacerbated during times of stress (Daniels et al., 2018).

Afferent alterations

An upregulation in afferent nerve activity is likely to be a contributor to this painful condition. In human bladder samples from IC/BPS patients, immunohistochemistry has identified a hyperinnervation of C-fibres in the sub-urothelium (Pang et al., 1995, Regauer et al., 2017). Interestingly, hyperinnervation in the sub-urothelium and expression of the p75 receptor in urothelial cells distinguishes IC/BPS from overactive bladder (Regauer et al., 2017). As TRPV1 channels are located on sensory nerves, significant increases in sub-urothelial TRPV1 nerve fibres have been reported in IC/BPS bladder tissue samples that have been positively correlated with the degree of inflammation, the production of NGF and pain and urgency scores (Liu et al., 2014).

Mast cells, when activated secrete pro-inflammatory and nociceptive mediators relevant to IC/BPS such as histamine, prostaglandin, cytokines and chemotactic factors which are suspected to sensitize and promote the release of neuropeptides from bladder afferent neurons producing painful symptoms (Erickson and Davies, 1998, Theoharides et al., 2001, Park and Bochner, 2011). In the rat bladder, it was found that acetylcholine and SP could activate mast cells, although, many substances can cause activation; these can include chemicals, viruses, adherent bacteria, free radicals, hormones, NGF and CGRP (Spanos et al., 1996, Theoharides et al., 2001). Mast cells are increased in the detrusor and sub-urothelium of IC/BPS patients and have been found in close association with nerves in the sub-urothelium (Pang et al., 1995, Theoharides et al., 2001, Regauer et al., 2017).

Other theories of pathogenesis

Changes in the structure of Tamm-Horsfall protein

Another proposed mechanism for the pathological event leading to IC/BPS is defective protective mechanisms failing to neutralize regular metabolic waste. Tamm-Horsfall protein (THP) is the most abundant protein found in human urine. It has been suggested to be a protective macromolecule that can bind to the potentially harmful toxic factors found in normal human urine before they bind to the urothelium disrupting the permeability barrier (Tamm and Horsfall, 1950, Parsons et al., 2000).

The protective biological effect of THP depends on sialic acid found within the protein (Parsons et al., 2007a). Although patients with IC/BPS had the same concentration of THP in their urine compared to their normal subjects, their THP was found to be qualitatively different, as they produced THP with reduced amounts of sialic acid (Parsons et al., 2007b).

Autoimmune and viral theories

Other theories have suggested IC/BPS has an autoimmune component with some bladder biopsies containing immune deposits along with fibrinogen in the bladder vasculature suggesting immunological injury to the blood vessels. No immune deposits were found in in the blood vessels of healthy control bladders. Interestingly, some cases of IC/BPS have responded to immunosuppressive therapy (Oravisto and Alfthan, 1976, Mattila, 1982). Although controversial, it has been suggested that the pathogenesis of IC/BPS could be of viral origins. Recent studies have identified evidence of polyomaviruria and Epstein-Barr virus (EBV) in approximately 50% of patients diagnosed with IC/BPS. When the patients were examined by the assignment of their disease, i.e., ulcerating vs. non-ulcerating, the ulcerative form of IC/BPS was significantly associated with polyomaviruria while almost 90% of ulcerating IC/BPS patients had been affected by EBV (Benjamin et al., 2015, Jhang et al., 2018b).

Treatment protocols

No single treatment currently works for all cases of IC/BPS, but most patients do eventually receive good symptom control. Accordingly, alternative treatments are used and trialled until the patient's symptoms are satisfactorily controlled (Erickson and Davies, 1998). The AUA

and EAU have recently updated IC/BPS management guidelines with a goal of maximizing symptom control and quality of life while minimizing patient burden and adverse effects. Listed in the tables below are conservative, oral, intravesical and surgical treatments that have been used to treat IC/BPS including recommendations in the guidelines put forward by AUA and EAU (Hanno et al., 2011a, Hanno et al., 2015, Engeler et al., 2018)

Conservative therapies

Conservative therapy is recommended for all sufferers of this condition and should always be considered alongside oral or more invasive therapies for IC/BPS. Conservative therapy revolves around education, physical therapy/massage, behaviour modification, stress reduction, manoeuvres that resolve muscular trigger points and dietary manipulation. Some patients have received a significant improvement in their IC/BPS symptoms just by managing diet and reducing stress (Hanno et al., 2011a, Bosch and Bosch, 2014, Cox et al., 2016, Engeler et al., 2018).

Oral therapies

Oral medications are used when more conservative treatment measures are insufficient although their efficacy to treat the individual remains unpredictable (Hanno et al., 2011a). **Table 1.2** lists oral medications that have been used to help manage the symptoms of IC/BPS including the AUA and EAU guidelines that grade their appropriate use. The AUA has divided treatments into first, second, third, fourth, fifth and sixth-line groups with the more conservative therapies listed as first-line treatments.

Table 1.2: Oral medications that have been used to treat IC/BPS along with their actions/side effects and guidelines by the AUA and EAU.

Medication	Its action/side effects	AUA guidelines	EAU guidelines
Oral Pentosanpolysulfate	Its proposed mechanism is replacing a deficient urothelial glycocalyx. Side effects are rare (gastrointestinal upset and alopecia).	2nd line	Treatment of significant value.
Amitriptyline	It has several actions that may benefit IC/BPS which include antihistamine, anticholinergic sedation and inhibition of nociception in the CNS. Side effects can be sedation, anticholinergic activity and weight gain	2nd line	Treatment of significant value.
Antihistamines	The H1 blocker hydroxyzine and H2 blocker cimetidine are sometimes used as mast cell activation may contribute to IC/BPS symptoms. Side effects are minimal.	2nd line	Hydroxyzine is a treatment of significant value.
			Cimetidine is a treatment with limited value.
L-Arginine	The mechanism is thought to increase NOS activity.	N/A	Treatment of limited value.
Calcium channel blockers	Its mechanisms may work by increasing bladder blood flow, inhibiting detrusor contractions or depressing the immune response. Side effects include hypotension, dizziness and oedema.	N/A	N/A
Drugs for neuropathic pain	These have been useful for other types of chronic pain.	N/A	Duloxetine is a treatment of limited value.
Analgesics	These can be helpful in returning to a normal lifestyle and include OTC, topical, non-narcotic and narcotic medications.	Should be continually assessed.	Should be continually assessed.
Immunosuppressant's	Seems to improve pain and frequency but has serious side effects.	5th line	Cyclosporin A, methotrexate and azathioprine are treatments of significant value.

SC = subcutaneous. OTC = over the counter, N/A = information not available.

approved treatment, of limited value/unapproved treatment.

(Erickson and Davies, 1998, Hanno et al., 2011a, Hanno et al., 2015, Cox et al., 2016, Engeler et al., 2018).

Intravesical treatment

Intravesical therapy is offered when more conservative measures have failed or in conjunction with oral and physical therapies (Moldwin et al., 2007, Engeler et al., 2018). Intravesical treatment involves the instillation of a therapeutic agent directly into the bladder via a urethral catheter. Intravesical treatments are advantageous from the point of view that the agent is localized to the bladder in high concentrations while minimizing side effects. Patient compliance is usually good, and the procedure can be performed at home by self-catheterization. Usually, the primary goal of intravesical treatment is to address neurogenic inflammation, hypersensitivity and replenishment of the GAG layer in the bladder. These treatments are often used in conjunction with physical and oral therapies. (Neuhaus and Schwalenberg, 2012, Cvach and Rosamilia, 2015) Quite often intravesical "cocktails" are used, combining groups of medications from the list below (Interstitial Cystitis Association, 2015). **Table 1.3** lists the preparations that have been used to intravesically treat symptoms associated with IC/BPS and include the AUA and EAU guidelines with their graded recommendations.

Table 1.3 The medications that have been used to intravesically treat IC/BPS including their action/side effects, AUA and EAU guidelines.

Medication	Its action/side effects	AUA guidelines	EAU guidelines
Dimethyl sulphoxide (DMSO)	Has multiple actions such as anti-inflammatory, analgesic, muscle relaxant, influences histamine released from mast cells.	2nd line	No longer recommended: insufficient evidence.
Heparinoids	Enhances the glycocalyx, has antioxidant activity and inhibits leukocyte migration and function.	2nd line	Treatment of value.
Hyaluronic acid/ Chondroitin sulfate	Replenishes the glycocalyx and thus the barrier function of the urothelium.	N/A	Treatment of value.
Pentosan polysulfate	Reduces urothelial permeability to the noxious components of urine.	N/A	N/A
Vanilloids (Capsaicin and Resiniferatoxin)	Desensitizes the bladder afferents.	Insufficient evidence/ investigational.	Insufficient evidence/ investigational.
Bacillus Calmette-Guerin	Its action is based on the autoimmune cause for IC/BPS.	Not recommended.	Treatment of limited value.
Disodium cromoglycate	Inhibits mast cells.	N/A	N/A
Lignocaine	Has a direct analgesic effect.	2nd line	Treatment of value.
Oxybutinin	The anticholinergic effect reduces bladder overactivity.	N/A	Treatment of limited value.
Clorpactin	Has antimicrobial activity that was originally used to treat TB.	N/A	Treatment of limited value.
Silver nitrate	Has an antiseptic effect.	N/A	N/A
Steroids	Used for their anti-inflammatory effect.	Long term not recommended.	Not recommended.
Doxorubicin	A cytotoxic drug and its action is uncertain.	N/A	N/A

N/A = information not available. TB=tuberculosis.

approved treatment, of limited value/unapproved treatment.

(Dawson and Jamison, 2007, Hanno et al., 2011a, Hanno et al., 2015, Engeler et al., 2018).

Interventional/surgical procedures

According to the EAU, IC/BPS is benign and does not shorten life. Therefore, surgical procedures should rank last (Engeler et al., 2018). **Table 1.4** lists the procedures that have been used to treat refractory IC/BPS and lists the AUA and EAU guidelines.

Table 1.4 The surgical procedures that are used to treat IC/BPS including the side effects and guidelines put forward by the AUA and EAU.

Procedure	Its action/side effects	AUA guidelines	EAU guidelines
Hydrodistension	Complications include a flare in symptoms, bladder rupture, and necrosis.	3rd line	Useful for diagnostic purposes only, limited therapeutic role.
Directly treating Hunner's ulcers	Complications include bladder perforation or fibrosis, hemorrhage and bowel injury.	3rd line	A treatment of value.
Sacral Neuromodulation	Potential side effects are failure to improve, painful stimulation, uncomfortable sensations, battery site pain, seroma, infection, lead migration and mechanical malfunction.	4th line	A treatment of value.
Botulinum toxin-A	Adverse events are uncommon; however, side effects can be UTI, hematuria, elevated post-void residual and temporary clean intermittent catheterization.	4th line	A treatment of value.
Radical Surgery	This should be considered as a last resort.	6th line	Last resort.

UTI=urinary tract infection, ☐ approved treatment.

(Hanno et al., 2011a, Hanno et al., 2015, Cox et al., 2016, Engeler et al., 2018).

Future therapy

Emerging investigational therapies for IC/BPS include hyperbaric oxygen, sildenafil, cannabinoids, intravesical liposomes, monoclonal antibodies and stem cell therapy (Kim et al., 2016, Cox et al., 2016).

1.6 Research hypothesis and specific aims

Overall hypothesis

Dimethyl sulphoxide, resiniferatoxin (RTX) and capsaicin are used clinically and experimentally as intravesical drug treatments for IC/BPS that come into close contact with the urothelium. Therefore, the overall hypothesis to be tested in these studies is that these treatments will have actions on the urothelium, detrusor and efferent nerves that can explain the outcome on bladder function and mechanisms adding to the current body of evidence. An additional hypothesis is that ethanol, the vehicle used to dissolve RTX and capsaicin, will also exert actions on these tissues that may contribute to the side effect profile of these drugs.

Specific aims

The effects of three intravesical treatments for IC/BPS (DMSO, RTX and capsaicin) and also ethanol (the vehicle for RTX and capsaicin) will be investigated in pig and murine models of intravesical treatment. The effects of treatment on a number of localized bladder responses will be examined including:

In the pig model

1. The release of ATP and ACh from the urothelium/lamina propria
2. The integrity of the urothelium.
3. Spontaneous activity of the urothelium/lamina propria and detrusor.
4. Contractile responses of urothelium/lamina propria, detrusor and intact tissue strips to cholinergic, purinergic and KCl stimulation.
5. The response of the detrusor to nerve-mediated stimulation including the purinergic and muscarinic contributions.
6. The relaxation response of the detrusor to adrenergic stimulation.

In the mouse model

1. The release of ATP and ACh on distension.
2. Compliance to bladder filling.
3. Spontaneous activity.
4. The pressure response to cholinergic, purinergic and KCl stimulation.
5. The pressure response to nerve-mediated stimulation including the purinergic, muscarinic and nitrergic contributions

1.7 Summary

Interstitial cystitis/bladder pain syndrome is characterized by pelvic pain, urinary frequency, urgency and nocturia in the absence of bacterial infection or any other identifiable pathology. Cystoscopically, IC/BPS can present with or without lesions. Multiple areas of the bladder are affected. In particular, defects to the barrier of the urothelium such as an altered GAG layer, urothelial tight junctions and adhesion proteins. These alterations potentially expose the underlying tissues to noxious components found within urine resulting in neurogenic inflammation, consequential irritative functioning, and aberrant signalling. Bladder pain syndrome has a female preponderance usually affecting mid-life. This disease impacts patient quality of life with a significant economic cost. As this condition is complex, treatment requires a multimodal approach consisting of conservative, oral, intravesical, interventional and surgical treatments to achieve symptom control and most importantly pain relief.

Intravesical treatments are recommended when more conservative treatment measures have failed. Intravesical dimethyl sulphoxide (DMSO) and vanilloids have been used to treat IC/BPS clinically and experimentally although their action on the bladder remains unclear. The use of vanilloids is currently not approved for treatment, and although DMSO is widely used and is approved by the AUA. Since 2015, the EAU has stated that there is insufficient current evidence for the efficacy of DMSO and therefore is no longer recommended as a treatment. Subsequently, an investigation of the effects that these compounds have on bladder function could provide better outcomes for the patient suffering from IC/BPS (Hanno et al., 2015, Engeler et al., 2018).

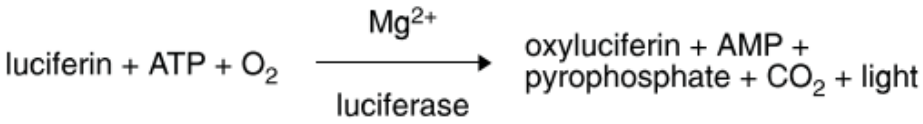
Chapter 2:
General methods

2.1 Assays used to detect urothelial mediators

Urothelial mediator's adenosine 5'-triphosphate (ATP), acetylcholine (ACh) and cytotoxicity marker lactate dehydrogenase (LDH) were analysed at various points in this study. The following commercially available assay kits were used according to their product protocols.

ATP determination kit

ATP release in response to treatment was quantified using Molecular Probes ATP determination kit (A22066). This is a bioluminescence assay that requires recombinant firefly luciferase and its substrate D-Luciferin to determine ATP. This assay is based on luciferases' requirement for ATP to produce light (Emission maximum ˜560nm at pH 7.8). The reaction is as follows:



Luminescence was measured by using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA). A standard curve using known concentrations of ATP was used for every plate of samples that were tested to determine ATP release potentially produced by treatment **(Figure 2.1)**.

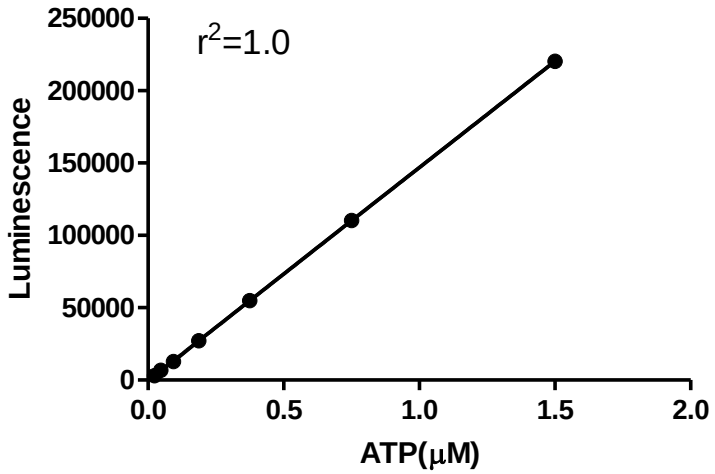


Figure 2.1: The luminescence of known concentrations of ATP plotted as the standard linear regression to analyse ATP found in our treated samples.

Amplex red acetylcholine/acetylcholinesterase assay kit

Acetylcholine release in response to treatment was determined by using Molecular Probes Amplex red acetylcholine/acetylcholinesterase assay kit (A12217). This is a fluorescence-based assay that works by using acetylcholinesterase (AChE) as the initial substrate. AChE converts ACh to choline, choline is then oxidised by choline oxidase to form betaine and H_2O_2. H_2O_2 in the presence of horseradish peroxidise reacts with Amplex Red reagent 1:1 generating a highly fluorescent product called resorufin that fluoresces between 571nm and 585nm **(Figure 2.2).** The fluorescent product was measured by using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA). A standard curve using known concentrations of ACh was used for every plate of samples that was tested to determine ACh release potentially produced by treatment **(Figure 2.3)**.

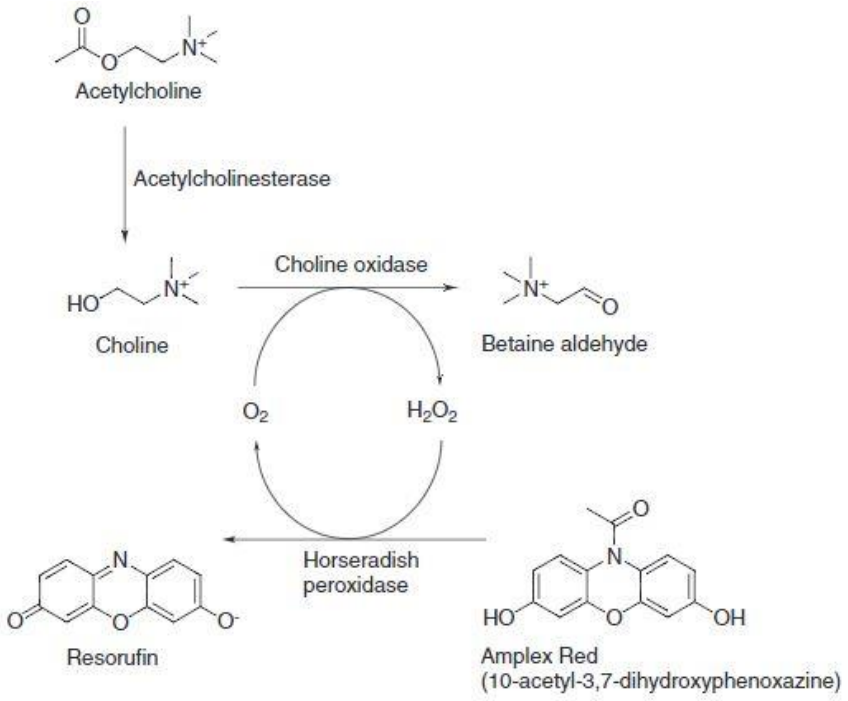


Figure 2.2: The enzymatic events that occur during the Amplex red assay that produce the highly fluorescent resorufin (reproduced with permission from the publisher, (Santillo and Liu, 2015))

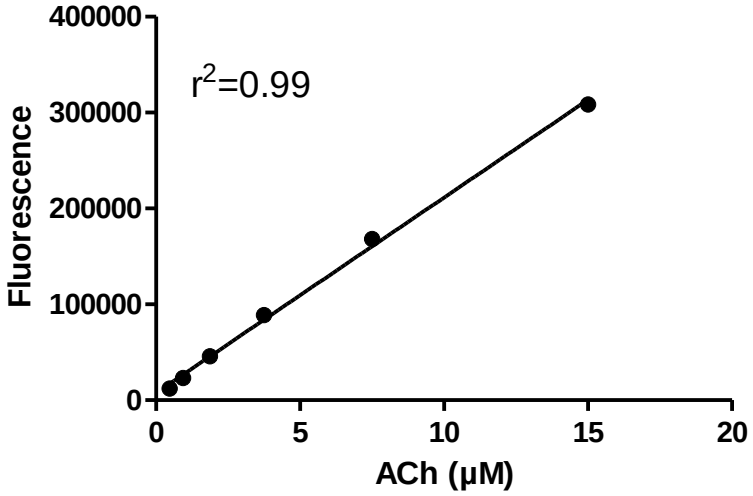


Figure 2.3: The fluorescence of known concentrations of ACh plotted as the standard linear regression to analyse ACh found in our treated samples.

LDH cytotoxicity assay kit

Lactate dehydrogenase (LDH) is a soluble enzyme that is released upon cell damage or lysis and is used as an indicator of cell membrane integrity. LDH release in response to treatment was assessed using Cayman Chemical Company LDH cytotoxicity assay kit (1000882) which measures LDH in a coupled 2-step reaction. Firstly, by oxidation of lactate to pyruvate, LDH catalyses the reduction of NAD^+ to NADH and H^+. In the second step, diaphorase uses NADH and H^+ to catalyse the reduction of the tetrazolium salt to highly coloured formazan that absorbs strongly at 490-520nm. The amount of formazan produced is directly proportional to the amount of LDH that has been released. The absorbance measurements were measured using a Microplate Absorbance Reader (Bio-Rad Laboratories, Inc. CA USA). A standard curve using known concentrations of LDH was used for every plate of samples that were tested to determine the LDH activity that was potentially produced by treatment **(Figure 2.4)**.

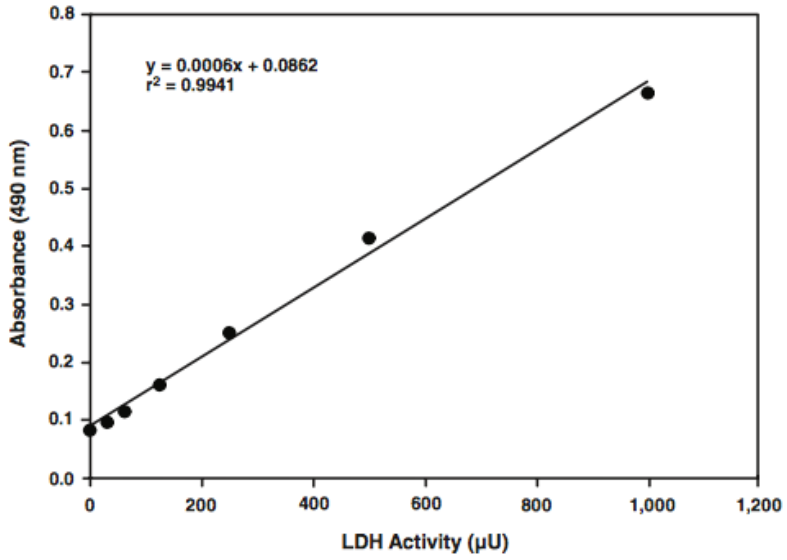


Figure 2.4: A typical standard curve measuring the absorbance of known LDH activity.

2.2 Histology

Histological studies were performed on whole pig tissue strips after luminal treatment with the drug of interest including a matched control. After dissection, tissues measuring approximately 7 mm long, 5mm wide and 5mm thick and tissues were processed by the following protocol.

1. Fixing the tissue – Tissues were fixed by placing them in isolated porous chambers that were placed into specimen jars that contained neutral buffered formalin (10%). The tissues were then refrigerated at 4°C for 24-hours.
2. Processing the tissue – The tissues were removed from the neutral buffered formalin and were embedded into paraffin blocks by placing them in the following solutions.
    a. 50 % ethanol for 30-minutes.
    b. 75% ethanol for 30-minutes.
    c. 90% ethanol for 30-minutes.
    d. 100% ethanol for 30-minutes.
    e. 1:1 xylene: ethanol for 15-minutes.
    f. 100% xylene for 15-minutes.
    g. 100% fresh xylene for 15-minutes.
    h. 1:1 xylene: liquid paraplast at 57°C for 30-minutes.
    i. Liquid paraplast at 57°C for 30-minutes.
    j. Final liquid paraplast at 57°C for 30-minutes.

Tissues were placed into aluminium moulds that were positioned before covering with liquid paraplast that were left to set for 12-hours.

3. Mounting tissue onto slides – Tissue was sectioned into 4 μm slices using an Accu-Cut ® SRM™ 200 Rotary Microtome (Sakura Finetek Europe B.V., The Netherlands). The sliced tissue was placed into a warm water bath at 48°C and was positioned onto a glass microscope slide and left to dry.
4. Preparation for staining – The slides which contained sections of tissue were placed in a heat box for 12-minutes and were rehydrated by placing the slides into the following solutions.
    a. 100% xylene for 3-minutes.
    b. Fresh 100% xylene for 3-minutes.
    c. 100% ethanol for 1-minute.
    d. Fresh 100% ethanol for 1-minute.
    e. 95% ethanol for 1-minute.
    f. 70% ethanol for 1-minute.
    g. Distilled water for 30-seconds.
5. Staining – The slides were placed in the following solutions.
    a. Haematoxylin stain (Mayers) for 4-minutes.
    b. Distilled water for 30-seconds.
    c. Acid alcohol (1%) for 30-seconds.
    d. Distilled water for 30-seconds.
    e. Scott's blue for 30-seconds.
    f. Distilled water for 30-seconds.
    g. Eosin stain for 2-minutes.
    h. Distilled water for 30-seconds.
6. Dehydrating and clearing for mounting – The slides were placed in the following solutions.
    a. 95% ethanol for 1-minute.
    b. 100% ethanol for 1-minute.
    c. Fresh 100% ethanol for 1-minute.
    d. Xylene for 1-minute.
    e. Fresh xylene for 1-minute.

7. Once dried, coverslips were fixed on top of the tissue section on the slide using a mounting medium, and the tissue sample was visualized by light microscopy (Infinity 2, Olympus, Tokyo, Japan).
8. Photographs were taken using Infinity 2 microscope camera (Olympus, Tokyo, Japan), Images were captured using Infinity Capture software (version 5.0.2 Lumenera Corporation, Canada).
9. Urothelial integrity from several bladder samples for each treatment group was measured using Image J software (http://imagej.nih.gov.ij).

2.4 Chemicals and pharmacological agents

Krebs-bicarbonate solution

Krebs-bicarbonate solution was used for all tissue experiments. The components of this solution are listed below **(Table: 2.1)**.

Table 2.1: The compounds used to make the Krebs-bicarbonate solution.

Compound	Formula	Molarity (µM) in 5 L dH_2O	Quantity (for 5 L)	Supplier	Catalogue #
Sodium Chloride	NaCl	118.41	34.6 g	Merck	S7653
Glucose	$C_6H_{12}O_6$	11.65	10.5 g	Merck	G0350500
Sodium bicarbonate	$NaHCO_3$	25.00	10.5 g	Merck	S5761
Potassium chloride	KCl	4.56	1.7 g	Merck	P9333
Magnesium sulphate	$MgSO_4$	2.41	1.45 g	Merck	M7506
Potassium dihydrogen orthophosphate	KH_2PO_4	1.18	0.8 g	Merck	RES20760-A7
Calcium chloride	$CaCl_2$	1900	9.5 mL	Merck	21115

Pharmacology

The drugs that were used for this project are listed in the table below. Stock solutions were made by using dH_2O or ethanol and were further diluted with Krebs solution or dH_2O where necessary. The concentrations of specific drugs that were used in this study are described in the materials and methods section for each chapter **(Table: 2.2)**.

Table 2.2: List of pharmacological agents, their action and supplier details.

Drug name	Formula	Its action	Supplier	Catalogue #
α,β – Methyladenosine 5'-triphosphate lithium salt	$C_{11}H_{18}N_5O_{12}P_3$	P2X Purinergic agonist	Merck	M6517
Adenosine 5′-triphosphate disodium salt hydrate	$C_{10}H_{14}N_5Na_2O_{13}P_3$	Purinergic agonist	Merck	A2383
Carbamoylcholine chloride (Carbachol)	$NH_2COOCH_2CH_2N(Cl)(CH_3)_3$	Muscarinic agonist	Merck	C4382
Isoprenaline hydrochloride	$C_{11}H_{17}NO_3$	β-AR agonist	Merck	I5627
Atropine	$C_{17}H_{23}NO_3$	Competitive muscarinic antagonist	Merck	A0132
N_ω-Nitro-L-arginine (L-NNA)	$C_6H_{13}N_5O_4$	Nitric oxide synthase inhibitor	Merck	N5501
Ethanol	C_2H_6O	Solvent	Merck	459844
Capsaicin	$(CH_3)_2CHCH=CH(CH_2)_4CONHCH_2C_6H_3$-4-(OH)-3-$(OCH_3)$	Vanilloid receptor agonist	Merck	M2028
Resiniferatoxin	$C_{37}H_{40}O_9$	Potent VR1 vanilloid receptor agonist	Merck	R8756
DMSO	$(CH_3)_2SO$	Solvent	Merck	D4540

Histology

The chemicals used for histology have been listed below **(Table: 2.3)**.

Table 2.3: The list of chemicals used for histological studies, its description and supplier details.

Product name	Description	Supplier	Catalogue #
Neutral buffered formalin (10%)	Tissue fixative	Fronine Laboratory Supplies	2518
Ethanol	Used for processing and staining	Merk	459844
Xylene	De-waxing and clearing	Fronine Laboratory Supplies	JJ028
Thermo Scientific™ Shandon™ Paraffin.	Embedding media	ThermoFisher Scientific	ALP502004
Haematoxylin (Mayers)	Stains tissue structures	Fronine Laboratory Supplies	II008
Hydrochloric Acid (Acid alcohol (0.5%) HCl + 70% EtOH)	Removes excess stain	Merk	320331
1% Alcoholic Eosin	Stains tissue structures	Fronine Laboratory Supplies	II017
Scotts Blue	Blueing solution for staining	Fronine Laboratory Supplies	II021
Ultramount No. 5	Used for coverslipping the slide	Fronine Laboratory Supplies	II065D
StarFrost® superclean, hydrophilic slides, ground edges 90°, white	Use for mounting tissue	Proscitech	G311SF-W
Coverslips 22x22	Specimen protection	Proscitech	G425-2222

2.4 Statistical analysis

A range of statistical tests were used throughout this study, and specific tests have been described in each of the investigational chapters where appropriate. Statistical significance was defined as $P<0.05$. All graphical analysis was completed by GraphPad Prism (Version 7.03) (Graph Pad software, San Diego, USA) and statistical analysis by Graphpad Instat (Version 3.10) (Graph Pad software, San Diego, USA).

Chapter 3:
The effects of dimethyl sulphoxide on the function of isolated pig bladder

3.1 Introduction

The pig as a model of bladder function

Due to a lack of freely available human bladder tissue, the pig bladder is often used and is considered to be more similar to the human bladder than any other laboratory species, sharing similar anatomy, physiology and pharmacology (Buckner et al., 2000, Dalmose et al., 2000, Sellers et al., 2000). Pig bladders were used in experimental chapters three, four and five and the following provides an overview of the similarities between pig and human bladder.

Parasympathetic supply and transmission

When the bladder is full, the parasympathetic nervous system promotes bladder emptying by contracting the detrusor and relaxing the bladder outlet. This is achieved by parasympathetic nerves co-releasing acetylcholine (ACh), adenosine 5'-triphosphate (ATP) and nitric oxide (NO). Generally, ACh and ATP promote contraction while NO relaxes the bladder outlet (Burnstock et al., 1972, Sibley, 1984, Kumar et al., 2004, Mundy, 2004, Bustamante et al., 2010).

Based on histochemical and immunohistochemistry work by Crowe and Burnstock (1989), the distribution of the parasympathetic nervous supply to the female pig bladder is similar to the female human bladder. However, in contrast to the human bladder, acetylcholinesterase (AChE) positive nerves are a little less abundant in the pig detrusor but are more dominant in the urethra, although, an excitatory cholinergic component has been demonstrated in pig detrusor (Sibley, 1984, Crowe and Burnstock, 1989). In addition to cholinergic fibres in the detrusor, cholinergic nerve terminals have also been observed around blood vessels in the sub-urothelium with single axons found beneath the urothelium (Lepiarczyk et al., 2011).

In many species, the detrusor contains mostly M2 and M3 receptors with a greater abundance of M2 receptors (Wang et al., 1995). The M2:M3 receptor ratio in the pig bladder is similar to the human bladder while the M3 receptor exclusively mediates the contractile response to pharmacological agents (Yamanishi et al., 2000). The contractile activity of the pig detrusor in response to carbachol (muscarinic agonist) relies on both intracellular and extracellular sources of Ca^{2+}. Though, it appears to be more reliant than human tissue on extracellular sources of Ca^{2+} via the L-type channel as nifedipine (L-type Ca^{2+} channel blocker) reduced the contractile

response to carbachol by 82% compared to a 25% reduction found in human detrusor tissue (Wuest et al., 2007).

Parasympathetic stimulation of healthy human bladder tissue produces contractions that appear to be exclusively cholinergic, however, parasympathetic stimulation of pig detrusor has a small component (≅ 15%) of atropine (muscarinic antagonist) resistant contractions that are potentiated by ATP (Sibley, 1984, Kumar et al., 2004). This component is significantly smaller than in other species studied and may represent a good model of some human bladder pathology where an increase in non-adrenergic, non-cholinergic (NANC) activity has been observed (Bayliss et al., 1999). Adenosine 5'-triphosphate co-released with ACh acts on purinergic receptors, namely P2X1 in the detrusor (Ralevic and Burnstock, 1998, Mundy, 2004). Moreover, purinergic P2X1 receptor mRNA transcripts are highly expressed in the pig detrusor which are undetected in the urothelial cell layers (Bahadory et al., 2013). Kumar et al (2004) has suggested a motor role for ATP in both the human and pig detrusor. However, the sensitivity to ATP is low.

In a similar fashion to the human bladder, relaxation of the bladder outlet in the pig is mediated by neurally released NO (Noda et al., 2002). Overall, Hashitani and Brading (2003) have concluded that pig and human bladders share similar electrical properties and have regarded the pig as a suitable model to investigate bladder function.

Sympathetic supply and transmission

The adrenergic nervous supply in the human has been described in the human bladder neck, urethra and trigone and is found very rarely in the detrusor (Gosling et al., 1977). In pigs, sympathetic supply is found more abundantly throughout the urinary tract including the detrusor and proximal urethra (Crowe and Burnstock, 1989). In addition, adrenergic nerve fibres have also been found surrounding the blood supply with some nerve terminals found in the sub-urothelium that extends to the urothelium (Lepiarczyk et al., 2011). The more abundant sympathetic supply in the pig bladder is supported by the earlier work of Larsen (1979) who found that isoprenaline (β-adrenoceptor agonist) was forty times more potent at relaxing the pig bladder detrusor compared to its ability in the human detrusor.

Sympathetically released noradrenaline acting on β-adrenoceptors in the detrusor promotes bladder relaxation during the storage phase of micturition while its action on the α-

adrenoceptors in the bladder base closes the bladder outlet (de Groat et al., 2015). Based on pharmacology, Larsen (1979) identified that the pig bladder was found to contain both α and β-adrenoceptors. The α-adrenoceptors were found mostly in the bladder neck while the β-adrenoceptors were found in the detrusor. Pharmacological studies later identified the $\alpha_{1A/L}$ -adrenoceptor was responsible for the sympathetic-mediated closure of the pig urethra (Bagot and Chess-Williams, 2006). Furthermore, relaxation of the pig detrusor occurs by a population of β_2-adrenoceptors which is not the case with human detrusor (Larsen, 1979). More recent work on the pig detrusor identified that both β_2 and β_3-adrenoceptors were active in promoting relaxation while that the β_3-adrenoceptors accounted for approximately 70% of the β-adrenoceptor population (Yamanishi et al., 2002). This differs slightly from intact pig bladder strips where β_3-adrenoceptors were found to be responsible for mediating much of the relaxation response (Masunaga et al., 2010). In contrast, β_3-adrenoceptor mRNA in the human detrusor is highly expressed (94.4%) and was pharmacologically found to evoke relaxation (Nomiya and Yamaguchi, 2003, Wuest et al., 2009).

Afferent supply and TRPV1 channels

The afferent system conveys sensations from the bladder (e.g. bladder fullness, pain etc.) back to the central nervous system to various coordinating centres that can determine bladder function (de Groat et al., 2015). Sensory afferent fibres are present in the pig bladder, but their distribution remains unclear (Pidsudko, 2014). However, neuropeptides associated with afferent fibres such as calcitonin gene-related peptide (CGRP), substance P (SP), Galinin and somatostatin have been identified in the pig bladder including the enzyme nitric oxide synthase (NOS). (Crowe and Burnstock, 1989, Pidsudko, 2014, Kozlowska et al., 2018). Immunohistochemistry has identified that a significant proportion of afferent nerves contain SP and transient receptor potential vanilloid 1 (TRPV1) channels and consistent with the human bladder, mRNA transcripts for the TRPV1 channel have been found in the pig bladder detrusor with higher expression found in the urothelial region (Sadananda et al., 2012, Kozlowska et al., 2018).

The urothelium

The role of the urothelium is to not only act as a barrier between urine and the underlying tissue but as a sensory transduction unit that communicates intra-luminal changes to the structures

beneath by expression of chemical mediators that may influence bladder function (Birder, 2010, Sellers et al., 2018).

Defined urothelial cell layers are present in human and pig urothelium including highly conserved uroplakins UPIa, UPIb, UPII and UPIII (Scheidegger, 1980, Jost et al., 1989, Wu et al., 1994). Immunofluorescence of the glycosaminoglycan (GAG) layer in the human and pig bladder has shown similar expression, function and distribution (Janssen et al., 2013). Similar to the human urothelium, the pig urothelium is the main source of ATP (86%) when it has been mechanically stretched and by electrical field stimulation (parasympathetic stimulation) (Kumar et al., 2004). In addition to ATP, the pig urothelium has also been found to be a source of PGE_2 and ACh (Smith et al., 2014, Kang et al., 2015). Both the human and pig urothelium have shown to produce an inhibitory effect on the detrusor by the release of urothelially derived inhibitory factor (UDIF) mediated by muscarinic stimulation (Hawthorn et al., 2000, Chaiyaprasithi et al., 2003, Propping et al., 2013).

Based on the above findings regarding the pig bladder, it has been selected for this study to examine what occurs during and after luminal treatment with dimethyl sulfoxide (DMSO), resiniferatoxin, capsaicin and their respective vehicles owing to its similarity to the human bladder in structure, function and availability. The results will likely closely reflect what occurs in the human bladder after the same treatment and will be useful to determine potential side effects and treatment outcomes. The more prominent NANC component found in pig bladders is additionally useful for describing potential outcomes for interstitial cystitis/bladder pain syndrome (IC/BPS) patients where this becomes more of a feature (Palea et al., 1993).

This section of the chapter to its conclusion is a pilot study pubished in BioMed Research International Volume 2014 (Smith, KJ., Chess-Williams, R., McDermott, C., Luminal DMSO: Effects on Detrusor and Urothelial/Lamina Propria Function. Article ID 347616, 8 pages. Online at http://dx.doi.org/10.1155/2014/347616).

3.2 Luminal DMSO: Effects on detrusor and urothelial/lamina propria function

Dimethyl sulphoxide has been used since the 1960's to treat the symptoms of interstitial cystitis/bladder pain syndrome IC/BPS (Stewart et al., 1967). Despite limited clinical trial data, Rimso-50 which is a 50% (v/v) solution of DMSO is used to relieve pain, and reduce the inflammation and voiding symptoms observed in patients with this condition (Moldwin et al., 2007, French and Bhambore, 2011). The exact mechanism of action of DMSO is not fully understood, however it is reported to cause mucosal damage and have analgesic, anti-inflammatory, bacteriostatic and muscle relaxant properties (Hohlbrugger and Lentsch, 1985, Moldwin et al., 2007, Kelada and Jones, 2007). Borzelleca et al. (1968) reported that 50% DMSO causes desquamation of the urothelium without altering the lamina propria (Borzelleca et al., 1968). Recently, studies have identified that DMSO directly affects cellular phospholipid membranes, with DMSO molecules found to occupy positions just below the lipid head groups acting as a spacer increasing average lateral distance favouring the entrance of water into the cell. Higher concentrations of DMSO directly increase lateral expansion of the cellular bilayer and have been known to cause destruction of cellular lipid bilayers (Gurtovenko and Anwar, 2007a, Gurtovenko and Anwar, 2007b). Patients commencing treatment with DMSO often experience an initial flare up of symptoms, which usually subsides after two weeks (Moldwin et al., 2007).

DMSO is highly permeable and will come into contact with the urothelium but possibly also the underlying lamina propria, detrusor smooth muscle and the nerves innervating the bladder wall. The urothelium forms a barrier, protecting underlying nerves and muscle from contents of the urine, however, the urothelium and lamina propria also play important roles in bladder sensation, with the urothelium releasing a number of mediators including ATP, ACh prostaglandin E2 (PGE_2), NO and an unidentified diffusible substance known as urothelial-derived inhibitory factor (UDIF) (Hawthorn et al., 2000, Birder et al., 2010b). These mediators

are involved in modulating sensory nerve activity and also detrusor function. DMSO has been reported to depress nitric oxide release from efferent nerves (Birder et al., 1997).

All five muscarinic subtypes are expressed in the human urothelium and stimulation of these receptors in the urothelium releases ATP, NO and UDIF (Birder and de Groat, 2007, Sellers and Chess-Williams, 2012). Bladder stretch during filling and activation of the urothelial muscarinic receptors (via ACh), triggers urothelial ATP release which is believed to be the source of primary excitation of the bladder afferents by acting on the P2X receptors (Birder et al., 2004, Hanna-Mitchell et al., 2007, Santoso et al., 2010). The urothelial P2X2/3 receptors have been implicated in the sensory role involved with micturition and also nociception in pathological states (Kumar et al., 2007, Sun and Chai, 2010). Myelinated Aδ afferent fibres are believed to be involved in the non-painful micturition reflex whereas high threshold unmyelinated C afferent fibres are activated in painful, pathological conditions.

Intravesical DMSO is used for the treatment of IC/BPS although patients initially experience a flare up of symptoms on commencement of treatment. Little is known regarding the effects of treatment on bladder function or the cause of the initial worsening of symptoms. As the urothelium comes into contact with the highest concentrations of DMSO during intravesical administration, changes in urothelial function may be involved in the drugs therapeutic actions but also initial worsening of symptoms. The aim of this study was therefore to investigate possible changes in urothelial/lamina propria and detrusor function using an *in vitro* model to simulate intravesical DMSO treatment.

3.3 Objectives

The purpose of this study was to investigate the effect that DMSO has on integrity and function of the pig bladder. Specific aims were;

1. To determine mediator release during luminal application of DMSO.
2. To examine the structure of the urothelium/lamina propria after treatment with DMSO.
3. To quantify mediators expressed by the urothelium/lamina propria in basal and stretch conditions following DMSO treatment.
4. To explore any contractile changes that may have occurred in the urothelium/lamina propria, detrusor and intact tissues as a result of treatment with DMSO.
5. To investigate the effect that DMSO has on efferent nerve mediated responses in the detrusor after treatment.

3.4 Materials and methods

Drugs, chemicals and reagents

Carbachol (carbamoylcholine chloride), ATP and DMSO were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Solutions were prepared in deionised water and further diluted in Krebs-bicarbonate solution.

Luminal treatment of porcine bladder with DMSO

Fresh bladders from Large White-Landrace pigs (six months old, 80Kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 118, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.15, KCl 4.7, KH_2PO_4 1.15 and D-glucose 11.7) The bladders were opened longitudinally and sheets of full thickness anterior wall from the dome region were set up in modified Ussing chambers. Separated gassed (5% CO_2/95% O_2) solutions bathed the luminal and serosal surfaces **(see Figure 3.1)**, allowing DMSO to be administered to the luminal surface only, with Krebs-bicarbonate solution bathing the serosal surface. The tissues were incubated at 37 ºC for 15 min with a therapeutic concentration (50% v/v) of DMSO applied to the luminal surface. Control bladders were incubated for 15min without the addition of DMSO. Incubation media was collected immediately after the 15-minute incubation to measure release of ATP, ACh and lactate dehydrogenase (LDH) from the luminal surfaces during the treatment. After the incubation (control or DMSO), tissue strips were then isolated and set up under 1g resting tension in organ baths containing Krebs-bicarbonate solution at 37ºC to allow the examination of tissue responses. Four sets of tissues were examined: -

(i) full thickness bladder wall with an intact urothelium and lamina propria
(ii) denuded detrusor strips with the urothelium and lamina propria removed
(iii) strips of urothelium and lamina propria for recording of tissue contraction and
(iv) strips of urothelium and lamina propria for the measurement of stretch-induced ATP and ACh release.

Functional organ bath studies

To assess the effects of DMSO on tissue responsiveness, contractile response to ATP (1 mM) and cumulative concentration-response curves to carbachol (1 nM – 10 µM) were obtained on tissues (i), (ii) and (iii). Isometric contractions of isolated tissue strips were recorded using a Powerlab data acquisition system (AD Instruments).

To investigate the effects of DMSO on nerve mediated responses, detrusor strips denuded of urothelium and lamina propria (tissues (ii) above) were set up under 1g resting tension in organ baths and electrically field stimulated via silver electrodes placed either side of the tissue. Tissues were stimulated at 1, 5, 10 and 20Hz using 5s trains of pulses (20v, 0.5ms pulse-width) delivered every 100s. Contractile responses and the release of mediators for tissues from DMSO pre-treatment bladders were compared with those of tissues from control incubated bladders.

Tissues (iv) above were used to examine the effects of DMSO on basal and stretch-induced release of mediators from the urothelium/lamina propria. These tissues were washed and 2min later a sample of the bathing medium was collected and frozen for later assay of mediators (basal release). The tissues were then stretched, increasing length by 50% and the bathing medium again collected and frozen for assay of mediators (stimulated release).

Measurement of ATP, ACh and LDH

ATP was measured using a luciferase-luciferin assay kit (Molecular Probes) according to the manufacturer's instructions. Luminescence was measured using a Modulus microplate reader (Promega). Acetylcholine was measured using a fluorescence-based Amplex® Red Acetylcholine Assay kit (Molecular Probes) according to the manufacturer's protocol. Fluorescence was measured on a Modulus Microplate reader (Ex. 540 / Em. 590nm). Leakage of LDH into the incubation media was measured using LDH Cytotoxicity assay (Cayman Chemicals). Absorbance was measured on a Modulus Microplate reader (490 nm).

Bladder histology

Sections of control and DMSO pre-treated intact bladder dome were fixed (4% neutral buffered formalin), processed and embedded in paraffin. Tissues were sectioned at 6 µm and placed on uncharged slides. Sections were stained using haematoxylin and eosin to assess urothelial integrity and examined using an Olympus CX31 microscope (Olympus Australia Pty. Ltd.)

equipped with an Infinity 2 camera and Infinity Capture software. Image J software was used to measure relative urothelial thickness in control and DMSO pre-treated tissues.

Data analysis and statistical procedures

Mean (± SEM) increases in tension induced by carbachol or electrical field stimulation were calculated. For responses to carbachol, individual –Log EC50 (pEC_{50}) values were determined from the concentration-response curves by use of GraphPad Prism 5 software (SanDiego, CA) and mean (± SEM) pEC_{50} values and maximum responses calculated. Similarly, for the mediator release study, mean (± SEM) concentrations were determined before and after stretch and data for DMSO and control pre-treated bladders compared. Data were analysed using a paired Student t-test or one-way ANOVA with Dunnett multiple comparisons test, using Graphpad InStat3 software (SanDiego, CA). Significance levels were defined as $p<0.05$.

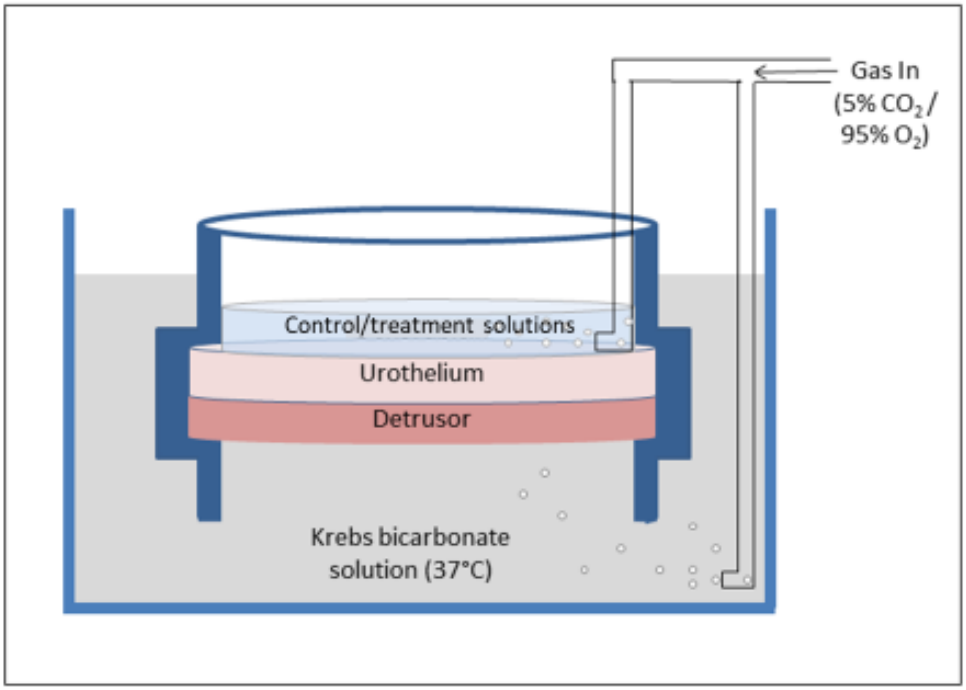


Figure 3.1: Schematic figure of the modified Ussing chamber. Full thickness sheets of bladder dome were sandwiched between two separated bathing solutions, each containing gassed (5%CO_2/95%O_2) Krebs-bicarbonate (serosal) or DMSO (urothelial) solution at 37ºC. Tissues were incubated with DMSO (50% v/v) or control solutions that were applied to the luminal side only for 15 min before isolation of the various tissues for pharmacological analysis.

3.5 Results

Mediator release during incubations

At the end of the treatment period the incubation medium was collected and assayed for ACh and ATP. Concentrations of ACh were significantly greater than those of ATP during both control (1.22 ± 0.05µM vs 0.010 ± 0.003µM, P<0.001, n=4) and DMSO incubations (21.3 ± 2.94µM vs 1.12 ± 0.09µM, P<0.001, n=4). The presence of DMSO during the incubation produced a significant increase in the levels of both mediators **(Figure 3.2 A&B)**, ACh levels rising 17-fold (P<0.001) and ATP levels rising by >100-fold (P<0.001). LDH (7 mU/mL) was also detected in luminal incubation medium from DMSO treated bladders with none detected in matched controls (data not shown).

Mediator release from the urothelium/lamina propria after incubation

Isolated strips of urothelium/lamina propria prepared from control incubated bladders released both ATP and ACh under basal and stretch conditions. There was a significant increase in ATP release in response to stretch **(Figure 3.2C)** in control tissues. However basal and stretch induced ATP release from DMSO treated tissues were not detected. ACh was released from control urothelium/lamina propria under basal conditions with no significant increase in response to stretch. ACh release from DMSO treated tissue was significantly reduced **(Figure 3.2D)**.

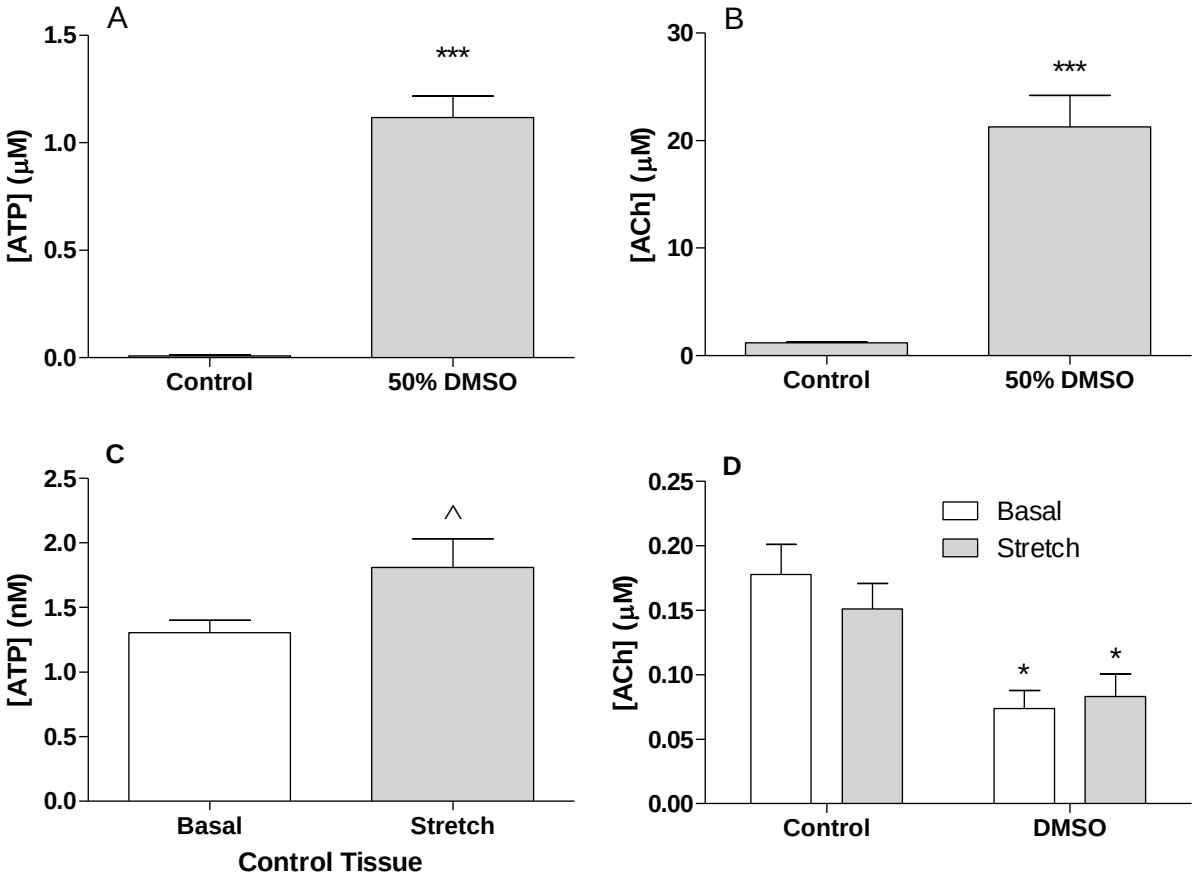


Figure 3.2: Effect of DMSO (50% v/v) on (A) ATP and (B) Ach release into incubation medium during bladder pre-treatment and also on subsequent basal and stretch-induced release of (C) ATP and (D) ACh from strips of urothelium/lamina propria. Data is represented as mean ± SEM (n=5). (*P<0.001, DMSO pre-treated vs control incubated bladders. ^P<0.05, control basal vs control stretch. *P<0.05, DMSO pre-treated vs control incubated tissues).**

Bladder histology

Representative H&E stained sections of control and DMSO pre-treated bladders (detrusor + urothelium/lamina propria) are shown **(Figure 3.3)**. Typical histological features were clearly identifiable in sections of control incubated bladder, with the urothelium and lamina propria thrown into folds and overlying a deeper smooth muscle layer. However, in DMSO pre-treated tissues, damage to the luminal layers was evident. Urothelial thickness was significantly reduced from 31.1 ± 1.48 µm in control tissues to 11.3 ± 0.45 µm in DMSO treated tissues ($n = 15$, $p < 0.001$). In addition, the folding of the urothelium/lamina propria observed in control bladders was absent from DMSO pre-treated bladders.

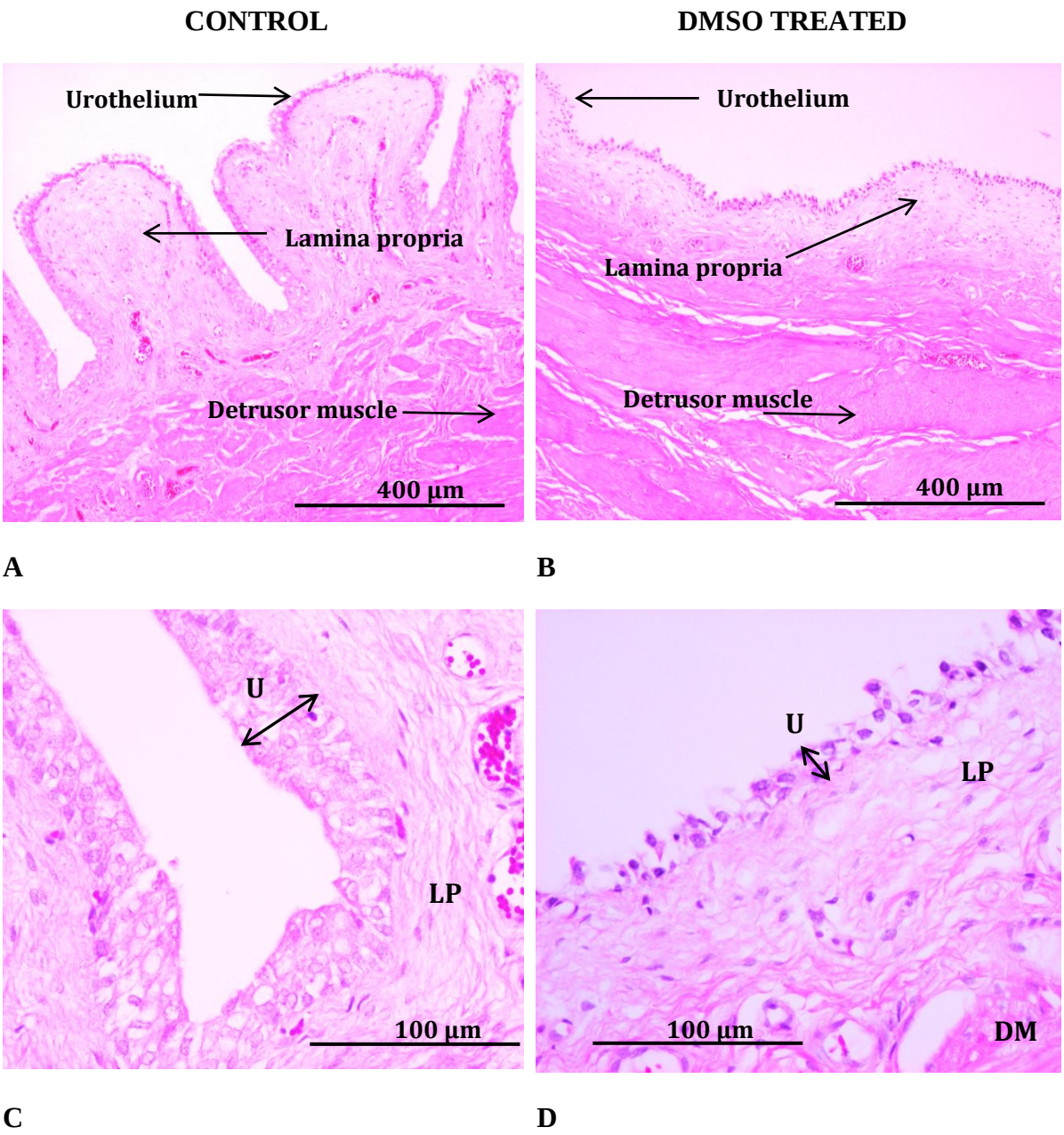


Figure 3.3: Hematoxylin and eosin (H and E) staining of control (A & C) and DMSO pre-treated (B & D) bladders. H and E staining at (A & B) 10X and (C & D) 40X.

Contractile responses following incubation with DMSO

Luminal pre-treatment of bladders with DMSO (50%) did not significantly affect detrusor, urothelial or intact tissue response to KCl or ATP (data not shown). Similarly, DMSO pre-treatment did not affect subsequent responses of isolated detrusor smooth muscle strips to carbachol **(Figure 3.4A)**, with both pEC_{50} values and maximum responses to carbachol being similar in muscle strips from DMSO and control pre-treated bladders **(Table 3.1)**. However, responses of urothelium/lamina propria strips and the responses of intact bladder strips (detrusor plus urothelium/lamina propria) to carbachol were enhanced after pre-treatment with DMSO **(Figure 3.4B&C, Table 3.1)**. The presence of the urothelium/lamina propria in the intact tissues significantly inhibited contractions of bladder strips **(Figure 3.4, Table 3.1)**. This inhibition was significantly ($P<0.05$) greater in control tissues (53 ± 7.8%) than in tissues from DMSO-pre-treated bladders (33 ± 4.1%).

Detrusor responses to electrical field stimulation (EFS) were frequency-dependent and contractions were increased in tissues from DMSO pre-treated bladders. Contractions to EFS were greater at all stimulation frequencies examined, the differences being statistically significant for the responses at 5Hz, 10Hz and 20Hz **(Figure 3.4D)**. In the presence of atropine (1 µM), detrusor contractions to EFS were depressed by 68 ± 10 % at 20Hz in control tissues ($P<0.001$). The inhibition of responses to EFS by this muscarinic antagonist was similar at all frequencies examined and was not altered significantly by DMSO pre-treatment (75 ± 6.1 % inhibition at 20Hz).

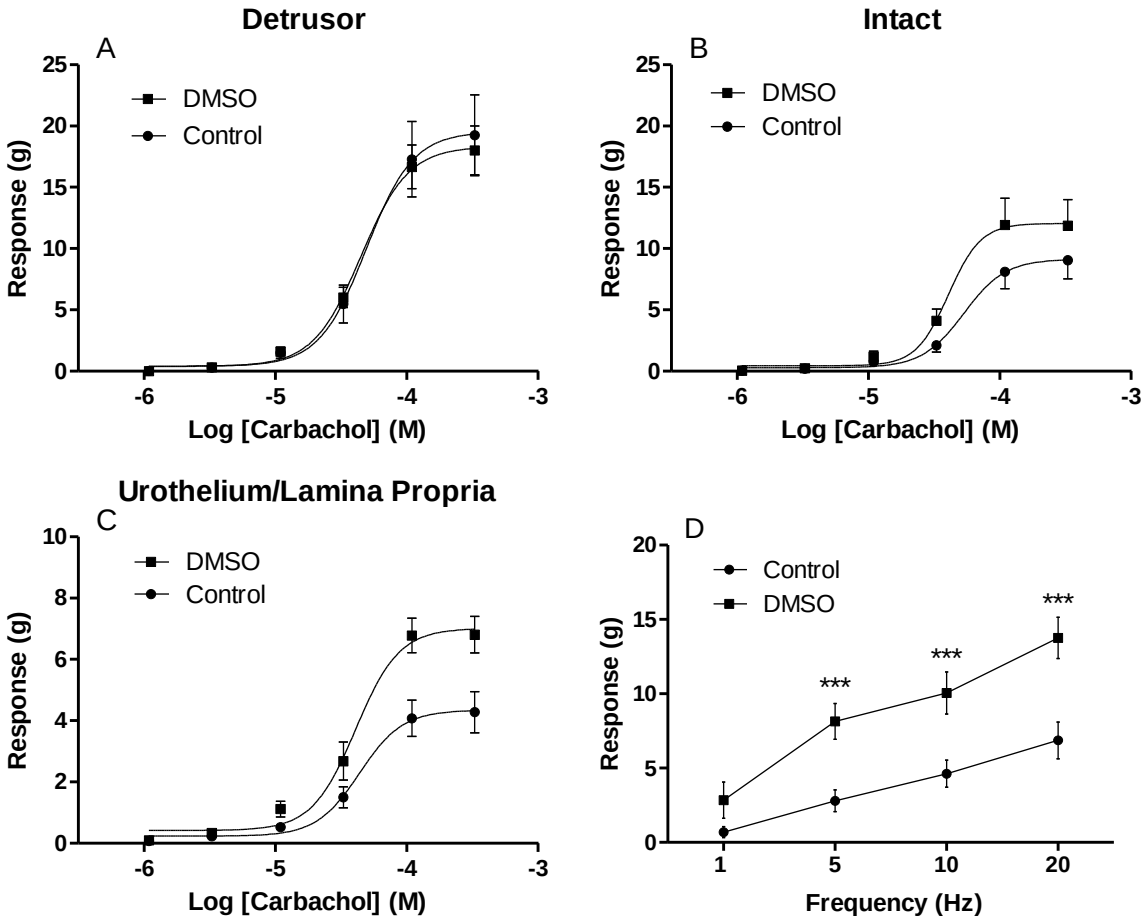


Figure 3.4: Cumulative carbachol concentration-response curves for DMSO (50% v/v) pre-incubated and control pre-incubated strips of (A) denuded detrusor, (B) intact tissue and (C) urothelium/lamina propria. Detrusor strip responses to EFS (D) are also shown. Data is represented as mean ± SEM (n≥ 4). ***P<0.001 compared to responses of control incubated tissues.

Table 3.1: Mean (±SEM) maximal responses (g) and pEC_{50} values for carbachol on tissue strips prepared from DMSO or control pre-treated bladders (n≥4). *P<0.05 and ***P<0.001 when comparing control vs. DMSO (50% v/v).

	Urothelium/lamina propria		Detrusor smooth muscle		Intact bladder strips	
Carbachol	Control	DMSO	Control	DMSO	Control	DMSO
Maximum response (g)	4.2±0.28	6.6±0.35 ***	17.8±1.4	17.1±0.7	8.3±0.63	11.5±0.94 *
pEC_{50}	4.14±0.09	4.4±0.08	4.37±0.12	4.39±0.07	4.35±0.14	4.45±0.52

3.6 Discussion

Although the exact mechanism by which DMSO relieves symptoms associated with IC/BPS is unclear, when applied to the human skin it penetrates rapidly and producing pharmacological effects such as anti-inflammation, analgesia, bacteriostasis (Shirley et al., 1978). For IC/BPS, DMSO is administered intravesically and due to the highly absorptive nature of DMSO, it is likely that not only the urothelium but the underlying lamina propria, detrusor smooth muscle and nerves innervating the bladder wall will also be affected by DMSO. However, it is the urothelial layer that comes in to direct contact with DMSO and is subject to the highest concentrations.

Treatment with DMSO is usually biweekly for 3- months and this has been found to be effective for approximately 16 to 72 months (Irwin, 2010). Previous studies have reported urothelial desquamation, mucosal damage and interference of cellular phospholipid membranes to be associated with the application of DMSO (Borzelleca et al., 1968, Hohlbrugger and Lentsch, 1985, Moldwin et al., 2007, Kelada and Jones, 2007, Gurtovenko and Anwar, 2007a, Gurtovenko and Anwar, 2007b). Chemical injury and subsequent loss of urothelial layers exposed to DMSO over a 3-month period may explain some of its effectiveness in treating IC/BPS as it has been reported that the removal of a diseased urothelium by laser treatment leads to non-recurrence of pain for 6-12 months (Birder et al., 2012). In the present study, loss of urothelial layers and mucosal folding were also detected following DMSO treatment. In addition, the detection of LDH in the luminal treatment effluent suggests that DMSO permeabilised the urothelial membranes causing leakage of cytosolic contents. The high levels of ATP and ACh detected in the treatment effluent may therefore reflect leakage of these mediators from the urothelium due to physical damage rather than enhanced levels of release.

High levels of ATP and ACh were observed during luminal treatment with DMSO, and urothelial mediator release post-treatment was also investigated. Following exposure to DMSO neither subsequent basal release nor stretch-induced release of ATP could be detected. ACh release was also significantly reduced following DMSO treatment. This is likely due to depletion of ATP and ACh stores during DMSO treatment and loss of urothelial cells from the mucosal surface. The inhibition of ATP release in response to stretch from urothelium treated with DMSO is consistent with previous reports which have identified a significant decrease in

ATP release from the urothelium treated with DMSO in response to stretch (Sun and Chai, 2002).

Augmented release of urothelial ATP and changes to urothelial purinergic P2X and P2Y receptor profiles are common features of patients suffering with IC/BPS (Birder and de Groat, 2007). It is well established that ATP, released in response to stretch, acts on afferent nerve $P2X_{2/3}$ receptors, playing a sensory role in the micturition cycle and also nociception in pathological states (Sun and Chai, 2010). Therefore, lower levels of urothelial ATP release after treatment with DMSO may be beneficial in correcting the augmented ATP release and the enhanced afferent nerve activity observed in IC/BPS.

It has been reported that in the porcine and human bladder the urothelium/lamina propria releases a factor that inhibits detrusor contraction (Hawthorn et al., 2000). This inhibitory effect of the urothelium was evident in tissues from both control and DMSO pre-treated bladders, but the inhibitory effect was significantly reduced following DMSO-pre-treatment. The consequences of this change are unknown, but a similar reduction in inhibitory mechanisms has been observed in the human neurogenic overactive bladder (Chess-Williams, 2009). Thus, the observed reduction in urothelial inhibition of the detrusor may contribute to the bladder overactivity observed after DMSO treatment. Surprisingly urothelial contractile responses to carbachol were enhanced following DMSO-pre-treatment. It has been suggested that this activity is responsible for correct folding of the urothelium on bladder emptying (Sadananda et al., 2008) or contractile activity of this layer may drive detrusor contraction (Moro et al., 2011) but the clinical relevance of this urothelial activity is currently unknown.

Detrusor responses to carbachol were not affected by DMSO pre-treatment suggesting that only minimal concentrations of DMSO have permeated to the deeper tissues. However, detrusor responses to EFS were altered by pre-treatment which indicates that the parasympathetic nerves in this tissue are more sensitive to the actions of DMSO than the detrusor muscle itself. Unexpectedly, response to EFS were enhanced by DMSO pre-treatment. Since detrusor responses to exogenous carbachol were unchanged, this suggests that DMSO increases neurotransmitter release to EFS. The enhanced responses may be the result of DMSO causing damage to the nerve terminals which may ease when depletion of these stores has been completed. This is consistent with previous research which also noted acute reflex firing of pelvic nerve efferent axons in response to DMSO (Birder et al., 1997). Atropine inhibited

responses to EFS similarly in control and DMSO pre-treated tissues, suggesting that the cholinergic contribution to neurotransmission did not change following treatment.

The acute administration of DMSO has been found to cause irritation (Tyagi et al., 2009) and the associated pain is reportedly caused by mast cell degranulation, in response to chemical injury which eases on depletion (Sokol, 2007). The increase in contractile responses and high levels of ATP are consistent with initial flare up in IC/BPS symptoms post-DMSO treatment.

In conclusion, this study demonstrates a physical and functional disruption of the bladder urothelium/lamina propria following luminal exposure to DMSO. There was a large release of mediators from the urothelium/lamina propria during treatment that are known to trigger micturition and initiate sensations of pain. These sensations would be greatly enhanced by the increases in muscarinic and purinergic receptors previously reported for patients with IC/BPS. The detection of LDH in the treatment medium suggests that release was due to permeabilization of the urothelial membranes rather than stimulated physiological release. These effects on mediators and also the reduced inhibitory role of the urothelium/lamina propria on detrusor contraction following DMSO pre-treatment, may contribute to the initial flare up in symptoms experienced by most patients following intravesical DMSO treatment.

Acknowledgements

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Chapter 4:
The effects of ethanol on the function of isolated pig bladder

4.1 Introduction

Before we begin to examine intravesical resiniferatoxin (RTX) and capsaicin we will initially address their common vehicle and solvent, ethanol. Ethanol is a popular co-solvent and penetration enhancer and is used in many transdermal and topical preparations. Bladder intravesical treatment with vanilloids such as capsaicin and RTX are usually dissolved in 30% and 10% ethanol in 0.9% saline solutions respectively (Chancellor and de Groat, 1999). Due to its structure and ability to attract polar and non-polar molecules, ethanol is thought to facilitate penetration of capsaicin and RTX by firstly disrupting the glycosaminoglycan (GAG) layer allowing passage of these compounds through cell-to-cell channels until they reach the lamina propria and capsaicin-sensitive fibres (Mishina et al., 1986, Chancellor and de Groat, 1999, Engler et al., 1999). This is supported by reports that maximum concentrations of capsaicin were achieved in human skin by using isopropyl alcohol as the vehicle compared to mineral oil or propylene glycol preparations (Pershing et al., 2004).

The mechanisms for how intravesical instillations of ethanol act in the bladder are uncertain. However, ethanol in the bladder produces its own range of side effects, namely inflammation. In human studies that have used intravesical 10% ethanol as a control, it has been reported that patients experienced pain and temporary vague discomfort at the time of instillation thought to be due to ethanol (Chen et al., 2005b, Payne et al., 2005). Intriguingly, similar studies have all reported improvements in various urodynamic parameters within the control groups receiving instillations of 10% ethanol in saline. These improvements were suggested to be due to a placebo effect as the investigators were uncertain of the impact that ethanol has on the bladder (Payne et al., 2005, Kuo et al., 2006, Silva et al., 2007). Cystoscopy findings by Chen et al. (2005b) on bladders affected by interstitial cystitis/bladder pain syndrome (IC/BPS) post instillation of vehicular 10% ethanol, found that after treatment 50% of patients experienced mild changes such as erythema, vascularization and glomerulation. Furthermore, spinal cord injured and normal rat bladders receiving instillation of 10% ethanol in saline observed significant decreases in voiding pressure after administration. After a period of 24-hours, a significant increase in bladder capacity was apparent in spinal cord injured rats. These effects disappeared gradually from day two and returned to baseline after three to four days which led to the speculation that 10% ethanol in saline is not an inert vehicle (Ost et al., 2003).

Concerning the intravesical use of 30% ethanol in saline solution in the bladder, one study found that the immediate side effects that were observed with instillation of capsaicin (solubilized with a 30% ethanol/saline solution) were no different from the vehicle control group (30% ethanol/saline) suggesting that the solvent is the source of irritation. The vehicle control group side effects of suprapubic pain, haematuria, urine leakage, hot flushes and autonomic dysreflexia were persistent and eventually resolved over a period of one to two weeks. Also observed was a slight increase in maximum cystometric capacity (MCC) of approximately 25 mL thirty days after instillation (de Seze et al., 1998). Similar findings on the irritative effects of intravesical administration of 30% ethanol have also been reported by Wiart et al. (1998). Histological samples from rat bladders treated with intravesical 30% ethanol have featured urothelial thinning and sub-mucosal oedema and significant disruption of the GAG layer (Byrne et al., 1998).

The GAG layer in the bladder may offer some protection as the intravesical instillation of 10-20% ethanol in rabbit bladders produced zero blood alcohol concentration 10-minutes after the procedure, although, this time frame may not be adequate to determine its true effect (Monga et al., 2001). Another study attempted to use ethanol as a measure of bladder permeability by observing Breathalyzer data after intravesical instillation of 10,15 and 20% ethanol in three patients diagnosed with IC/BPS. During the dwell time of 30-minutes, no detectable levels were reported (Gordon et al., 2003).

Higher concentrations of ethanol have been used in the bladder. However, these were not delivered intravesically but rather, injected sub-cutaneously as it is known in other tissues for its ability to degenerate nerve fibres (Harris, 1940). Based on these findings, concentrations of 50% have been injected into the sub-trigonal region of the bladder to treat detrusor hyperreflexia. Overall, the majority of subjects converted to detrusor stability remaining dry or vastly improved at twenty-four months follow-up. Complications included fistula (Harris et al., 1988).

The vast majority of studies on the bladder use low concentrations of ethanol ranging from 0.1-3% as they can relate them to clinically relevant blood alcohol concentrations as a result of consumption. (Trevisani et al., 2002, Malysz et al., 2014). The immediate effects of ethanol on rat and rabbit bladders has been examined by adding increasing concentrations of ethanol to the bathing medium containing bladder tissue strips and examining the following contractile

response to electrical field stimulation (EFS), bethanechol (muscarinic agonist) and adenosine 5'- triphosphate (ATP). Rat bladder strips bathed in 3% ethanol had significantly reduced the responses to EFS, carbachol but not to potassium chloride (KCl) while lower concentrations were of no effect (Levin et al., 2005). Rabbit bladders demonstrated decreased resting tension and contractile response to bethanechol (muscarinic agonist) and ATP in a dose dependent manner ranging from 0.5%-3% while KCl mediated contraction was suppressed by 3% ethanol (Ohmura et al., 1997).

Detrusor muscle cells (DMC) express multiple receptors and ion channels that regulate bladder function, these include many potassium (K^+) channels such as large-conductance voltage – and calcium (Ca^{2+}) activated K^+ channels (BK) and L-Type voltage-dependent Ca^{2+} channels (VDCC). Generally, as Ca^{2+} entry via the L-type VDCC's is responsible for the initial depolarizing action of the DMC's, the K^+ channels shape the action potential by limiting Ca^{2+} entry repolarizing the cell causing DMC relaxation **(Figure 4.1)** (Petkov, 2011).

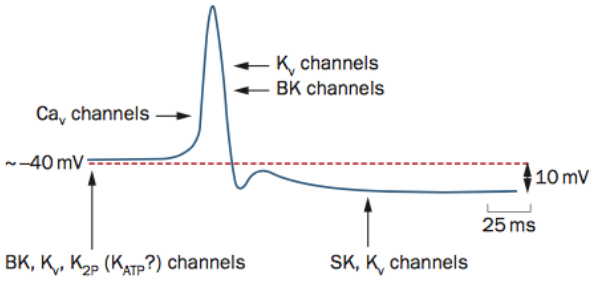

Figure 4:1: The various roles that K^+ channels have in determining resting membrane potential in detrusor smooth muscle (reproduced with permission from the publisher, (Petkov, 2011)).

The addition of 0.1-0.3% ethanol to guinea pig DMC's directly enhanced the activity of big potassium (BK) channels promoting detrusor relaxation. At higher concentrations of >0.3%, the L-type VDCC current was additionally inhibited by further decreasing detrusor activity. Furthermore, increasing concentrations of ethanol (0.01-1%) applied to detrusor strips attenuated amplitude and muscle force in spontaneous phasic contractions in a concentration-dependent manner (Malysz et al., 2014).

Ethanol on different tissues such as liver cancer cell lines and normal rat hepatocytes experienced total cytotoxicity 15-seconds after exposure to 30% and 40% ethanol. Cells that were exposed to concentrations of 15-20% ethanol produced cytotoxicity after 5-minutes, cells

exposed to 10% ethanol became cytotoxic after 50 mins while 5% had negligible effect (Tapani et al., 1996). Ethanol-induced cytotoxicity in cultured human skin cells was found to be dose and time-dependent. A concentration of 3.1% over a period of 24-hours increased the release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) that was implicated to be responsible for cellular damage and increased apoptosis (Neuman et al., 2002).

On a cellular level, lower and presumably non-cytotoxic levels of ethanol appear to activate transient receptor potential vanilloid (TRPV)1 channels similarly to capsaicin and RTX that will be discussed in more detail the following chapters. Using ethanol concentrations ranging from 0.1-3%, sensory nerves of the rat dorsal spinal cord, oesophagus and skin produced a concentration-dependent release of substance P (SP) in response to ethanol, presumably by the activation of TRPV1. Pre-treatment with capsaicin (desensitizes sensory neurons), capsazepine (TRPV1 antagonist) or removal of extracellular Ca^{2+} abolished this response. In dorsal root ganglion (DRG) and trigeminal rat sensory neurons including human TRPV1- expressing HEK293 cells, ethanol caused a concentration-dependent increase in cytosolic Ca^{2+}. Ethanol enhanced the Ca^{2+} response to capsaicin in TRPV1- expressing HEK293 cells increasing both the potency and efficacy although ethanol did not enhance the Ca^{2+} response to carbachol (Trevisani et al., 2002).

4.2 Objectives

The purpose of this study was to investigate the effect of the vehicle ethanol (10% and 30% (v/v)), relevant to the concentrations that are used to solubilize RTX and capsaicin respectively, has on integrity and function of the pig bladder. Specific aims were;

1. To quantify urothelial ATP, acetylcholine (ACh) release during luminal application of 10% and 30% ethanol.
2. To examine the structure of the urothelium/lamina propria after treatment with 10% and 30% ethanol.
3. To quantify mediators released by the urothelium/lamina propria in basal and stretch conditions after treatment.
4. To explore changes in contraction/relaxation responses that may have occurred in the urothelium/lamina propria, detrusor and intact tissues as a result of luminal treatment with 10% and 30% ethanol.
5. To investigate the effect that 10% and 30% ethanol has on efferent nerve-mediated responses in the detrusor after treatment.

4.3 Materials and methods

Animals

Fresh porcine bladder tissue from mature sows (>1-year-old) were retrieved from the local abattoir (Highchester Meats, Beaudesert, Queensland) and were immediately placed in ice cold Krebs bicarbonate solution (NaCl 118.4mM, NaHCO3 24.9mM, KCl 4.7mM, CaCl2 1.9mM, MgSO4 1.2mM, glucose 11.7mM) for transport back to lab facilities.

Tissue Preparation

After the urethra, ureters and excess tissues were removed from the bladder, the bladder was opened by dissection from base to dome and was laid flat on a dissection board. Whole bladder sections from the dome were isolated and were mounted in modified Ussing chambers **(see chapter three, Figure: 3.1)** containing gassed ($5\%CO_2/95\%O_2$) Krebs-bicarbonate solution and kept at 37°C as described by Smith et al. (2014). A 10% or 30% ethanol (v/v) in 0.9% saline solution was prepared and placed on the luminal side of the bladder tissue to incubate for 30-minutes reflecting clinical intravesical treatment. For each ethanol treatment, a matched control incubation (0.9% saline) was also conducted.

Sampling of effluent

After luminally treating the tissue for 30-minutes, a sample of the luminal medium was collected and was stored in a -30°C freezer for later analysis of urothelial ATP, ACh and lactate dehydrogenase (LDH) activity using commercially available kits (described in chapter two). The remaining treatment effluent was discarded.

Organ bath preparation

The tissue was then retrieved from the Ussing chambers and was washed with warm **(**37°C) Krebs-bicarbonate solution. Ethanol and control pre-treated tissues were sectioned into three strips each including a urothelium/lamina propria strip that was dissected from an intact strip, a denuded detrusor strip and an intact strip. Each strip was anchored in an individual organ bath containing gassed ($5\%CO_2/95\%O_2$) Krebs-bicarbonate solution kept at 37°C. In the organ baths, the tissues were connected to an isometric force transducer, and tension was recorded by a Powerlab 8/30 recording system (ADInstruments Ltd.) which was analysed by Lab Chart

(version 7.0.3) software (ADInstruments Ltd.). In preparation for the following experiments, the tissues were washed every 15-minutes and were allowed to equilibrate for 1-hour under a resting tension of 150 mN **(Figure:4.2)**.

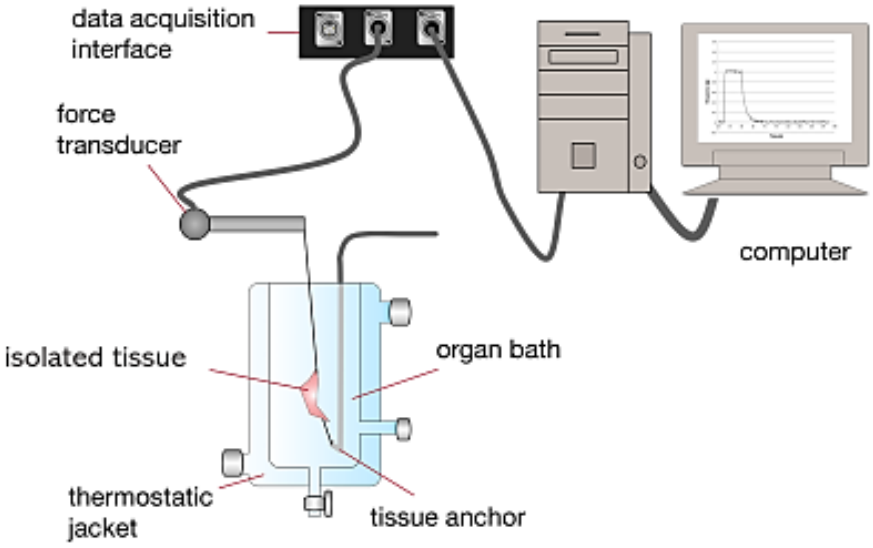

Figure 4.2: Illustration of an individual organ bath containing either an isolated strip of urothelium/lamina propria, detrusor or intact pig tissue. These tissues were anchored to the bath and were connected to an isometric force transducer to record changes in tension to experimental protocols. (Adapted from (Prince, 2015), This work is licensed under the Creative Commons Attribution-NonCommercial 2.0 UK: England & Wales License, https://creativecommons.org/licenses/by-nc-nd/2.0/uk/).

Spontaneous activity

After the equilibration period (1 hr), the spontaneous activity of the urothelium/lamina propria, and detrusor was investigated. Amplitude was recorded from peak to trough of a contraction and frequency was recorded as contractions that exceeded 30% of the peak amplitude per minute. Adapted from (Imai et al., 2001).

Functional studies using pharmacological agents

To begin the functional studies, both control and treated tissues were exposed to ATP (1 mM) and KCl (60 mM) to examine purinergic component and non-receptor mediated muscle contractile activity respectively. After peak contraction was observed following ATP, the

tissues were washed 2-3 times and were allowed to rest and re-equilibrate for 15-minutes before KCl was added to the organ bath. After a contractile plateau had been achieved in response to KCl, the tissues were washed another 2-3 times and allowed to re-equilibrate for another 15-minutes. Following on from ATP and KCl, a cumulative concentration curve was created in response to the muscarinic agonist carbachol (10 nM – 100 μM). After the maximal dose and peak response to carbachol had occurred, detrusor tissues were washed every 15-minutes and allowed to re-equilibrate for another hour before beginning a cumulative concentration curve in response to the β-adrenoceptor agonist isoprenaline (70 pM – 70 μM). Before recording the relaxation response to isoprenaline, the tissues were initially pre-contracted with 30 μM carbachol (submaximal dose) and allowed to plateau for approximately 20-minutes.

The recorded responses of each tissue were taken by measuring the change in tension from base line recordings before the addition of agonists. The changes in tension produced by the treated bladder strips were compared to their matched control strips.

Electrical field stimulation (EFS)

For nerve stimulation studies, strips of ethanol and control pre-treated detrusor strips were isolated and anchored in organ baths as described previously. Following equilibration, the strips were then electrically stimulated (20V, 1 ms pulse-width, 5 s train delivered every 100 s) to assess the nerve-mediated contractile responses in the detrusor. The stimulatory frequencies consisted of 1 Hz, 5 Hz, 10 Hz and 20 Hz and the subsequent contractile responses were recorded and assessed. These same frequencies were repeated in the presence of 1μM atropine and 10 μM α,β mATP to investigate the muscarinic and purinergic contributions.

Urothelial mediator release

In a separate experiment, to assess urothelial mediator release under basal resting conditions and stretch conditions, three strips each of ethanol and control pre-treated isolated urothelium/lamina propria tissues were tied together and anchored in organ baths as described above. Following the equilibration period, 3 mL of warm Krebs-bicarbonate solution was added to the bath for a 2-minute period. After the 2-minute period, samples of Krebs surrounding the tissue were taken to represent bladder basal resting conditions. Immediately after collection, the bath was drained, and another 3 mL of warm Krebs-bicarbonate solution was added to the bath. Over another 2-minute period, the tissues were stretched from 150 mN to 1.5 N simulating the stretch that occurs during bladder filling. After the stretch was complete,

samples were taken from around the tissue to assess mediator release in stretched conditions. All samples were stored in a -30°C freezer until required for further analysis of ATP and ACh release using commercially available kits (described in chapter two).

Histology

Following luminal treatment described above, intact bladder strips measuring approximately 1-2 mm wide were isolated and placed into neutral buffered formalin (10%) at 4°C for 24-hours. Tissues were then processed into wax blocks and stained with hematoxylin and eosin. Light microscopy was used to assess any alteration to urothelial integrity that may have occurred as a result of treatment. The protocol and materials are described in chapter two.

Statistical Analysis

For these studies, data were expressed as mean ± standard error of the mean (SEM) and was analysed by two-tailed T-Tests using Graphpad Instat (version 3.10) (Graph Pad software, San Deigo, USA). Curve analysis was performed with multiple comparisons F-test using GraphPad Prism (version 7.03) (Graph Pad software, San Deigo, USA). Significance was defined as *$P<0.05$, **$P<0.01$, ***$P<0.001$.

4.4 Results

Effect of luminal ethanol on the release of chemical mediators and the integrity of the urothelium/lamina propria

The immediate effect that luminally applied ethanol had on the urothelium was determined by quantifying urothelial mediators ATP, ACh and LDH activity (as a marker of loss of membrane integrity) in the treatment effluent that was in direct contact with the urothelium. To rule out any interference of the treatment medium with the assay reagents, additional standard assay curves were created using the appropriate treatment medium.

While tissues were being treated with 10% ethanol, there was a significant increase in ATP release (0.2±0.004 µM, $P<0.05$, n=8) compared to control treated tissues (0.005±0.002 µM, n=8) **(Figure 4.3A)**. A significantly more robust increase in ATP was found while treating tissues with 30% ethanol (30% EtOH 1.7±0.2 µM vs. control 0.01±0.001 µM, $P<0.001$, $n\geq7$) **(Figure 4.3B)**. In comparison to controls, there were no changes to ACh release while tissues were being treated with 10% ethanol (**Figure 4.3C)**, however, a significant reduction occurred in tissues treated with 30% ethanol (30% EtOH 0.2±0.2 µM vs. control 2.6±0.4 µM, $P<0.001$, n=8) **(Figure 4.3D)**. LDH activity was significantly enhanced by approximately 3-fold while tissues were being treated with either 10% or 30% ethanol **(Figure 4.3E&F)**.

To assess if ethanol had any damaging effects on the structure of the urothelium, histological analysis using hematoxylin and eosin staining of the intact tissue, with a focus on the urothelium/lamina propria, was compared between all pre-treated groups. Hematoxylin and eosin staining of the control pre-treated sections revealed characteristic histological features such as several layers of urothelial cells **(Figure 4.4C)** and a folded urothelium/lamina propria which overlays the detrusor **(Figure 4.4A)**. For tissues that had been pre-treated with 10% ethanol, there appeared to be some deficiencies in adhesion properties of the urothelial basal cells to the lamina propria compared to control treated tissues **(Figure 4.4E&F)** although measurement of the urothelial thickness revealed no alteration **(Figure 4.5A)**. For tissues that were pre-treated with 30% ethanol, clear and visible sloughing of the urothelial cells had occurred that extended to the basal cells **(Figure 4.4H&I)**. Measurement of the remaining urothelial layer in tissues pre-treated with 30% ethanol was significantly reduced when

compared to control treated tissues (30% EtOH 17.2±1.1 µm vs. control 69.4±6.4 µm, P<0.001, n=6) **(Figure 4.5B)**.

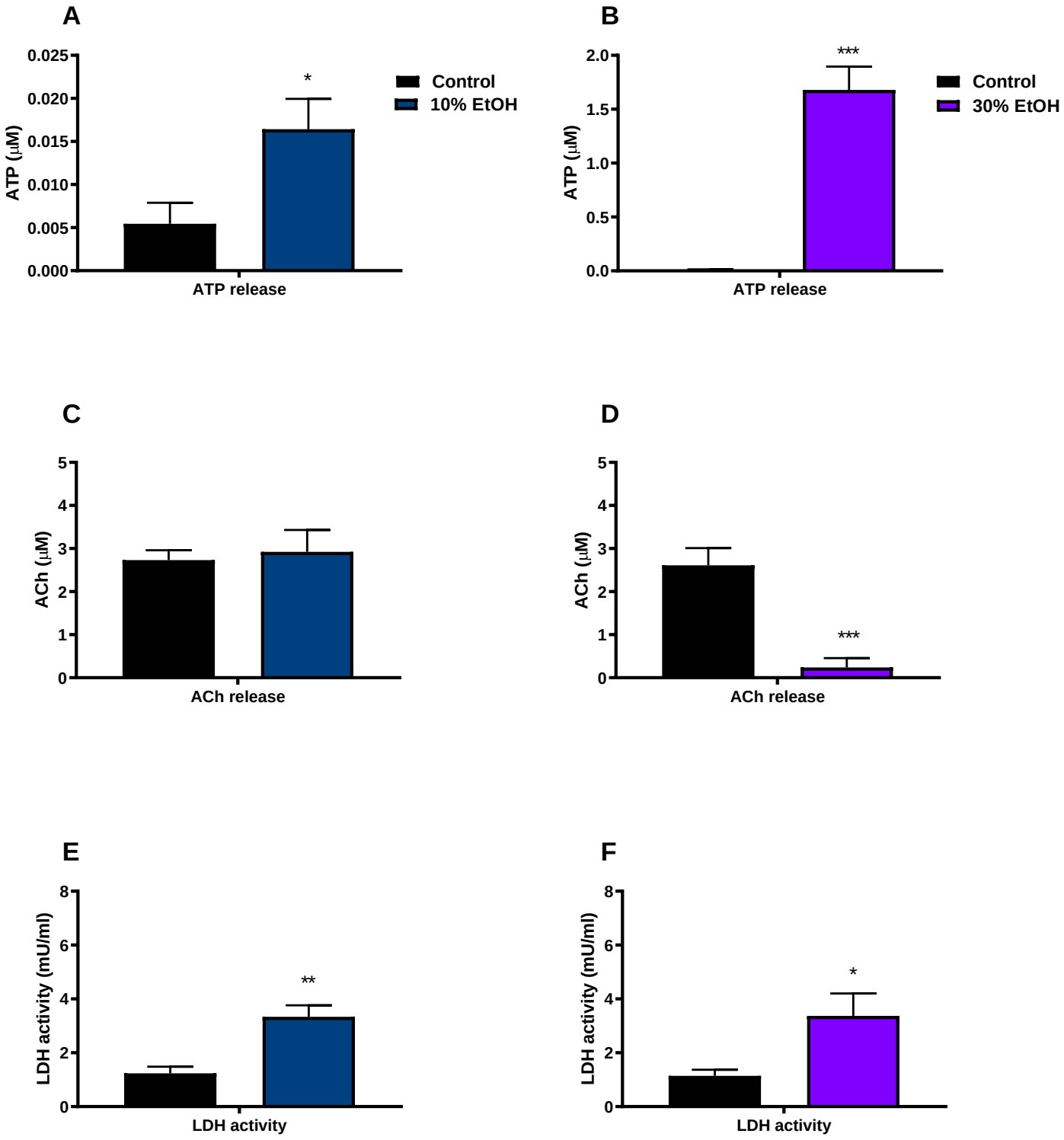

Figure 4.3: ATP release (A&B), ACh release (C&D) and LDH activity (E&F) in samples of the treatment effluent collected following luminal treatment of pig bladder tissues with control solution (0.9% saline), 10% EtOH (A,C&E) or 30% EtOH (B,D&F). Data is represented as mean ± SEM (n≥6) analysed by an unpaired two-tailed t-test (*P<0.05, **P<0.01, ***P<0.001 control vs 10% or 30% EtOH).

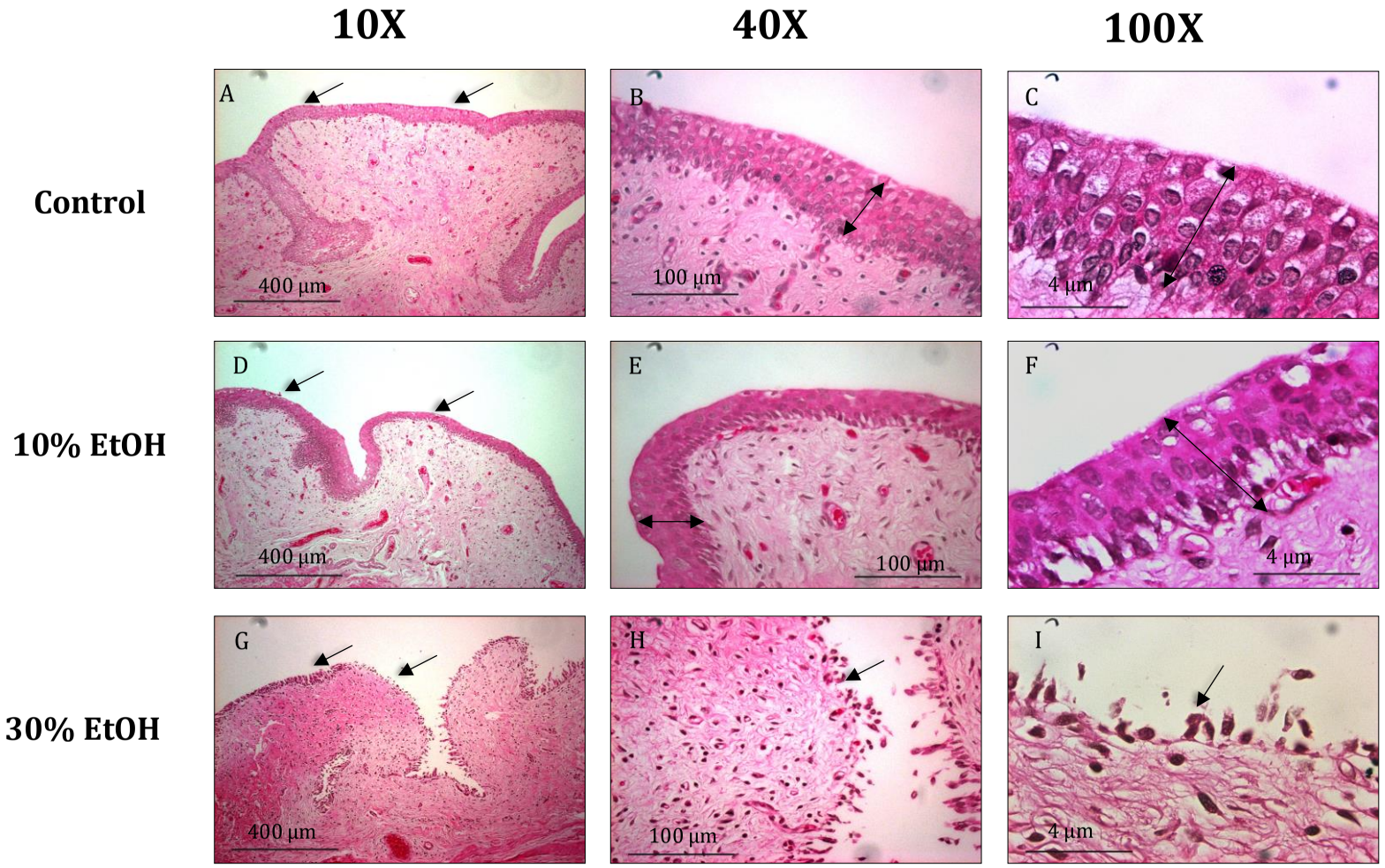

Figure 4.4: Histological sections of intact pig bladder tissue (H&E stain) with a focus on urothelium/lamina propria after luminal pre-treatment with control solution (0.9% saline),10% EtOH or 30% EtOH. A,B & C represent the control treated tissues at 10,40 and 100x magnification. D,E & F represent tissues treated with 10% EtOH at 10,40 and 100x magnification. G,H & I represent tissues treated with 30% EtOH at 10,40 and 100x magnification. Black arrows indicate the thickness of the urothelium.

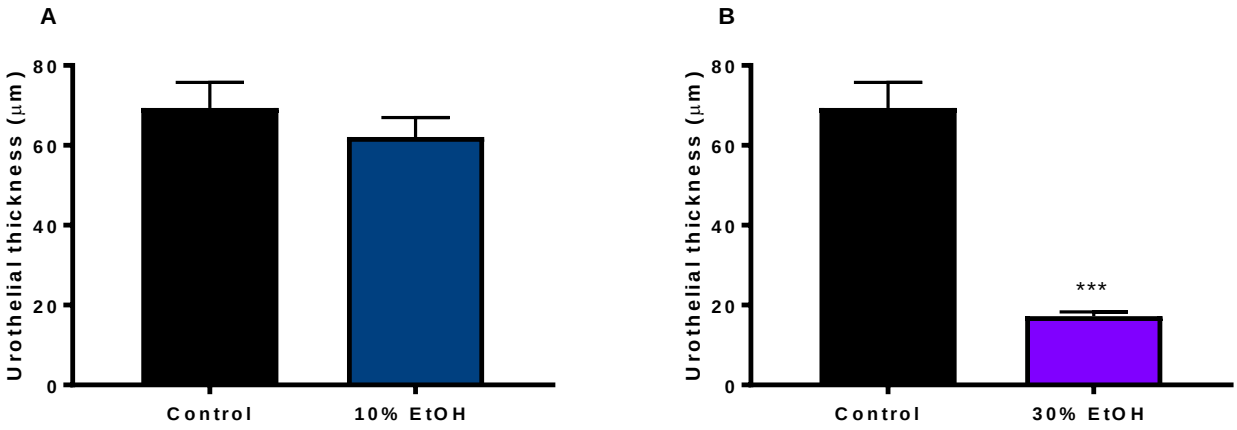

Figure 4.5: Urothelial thickness (µm) following luminal pre-treatment with control solution (0.9% saline), 10% EtOH (A) or 30% EtOH (B). Data is represented as mean ± SEM (n=6, taken from an average of 18 measurements), analysed by an unpaired two-tailed t-test (*P<0.001, control vs. 10% or 30% EtOH).**

Effect of ethanol pre-treatment on basal and stretch-induced mediator release

To determine if luminal pre-treatment with ethanol had impacted the release of mediators from the urothelium/lamina propria, ATP and ACh release was investigated from isolated strips of urothelium/lamina propria under basal and stretched conditions.

For all control pre-treated tissues, ATP release between basal and stretch conditions was not significantly different **(Figure 4.6A&B)**. However, ACh release was significantly enhanced by mechanical stretch (results not shown), **(Figure 4.6C&D).**

Following pre-treatment with 10% or 30% ethanol, the basal release of ATP was significantly reduced by 60% compared to the control treated tissues **(Figure 4.6A&B)**. In response to stretch, ATP release was also significantly reduced for same tissues pre-treated with 10% or 30% ethanol by 80% and 60% respectively **(Figure 4.6A&B)**. The basal release of ACh was abolished in tissues as a result of ethanol pre-treatment **(Figure 4.6C&D)** while there were no changes to stretch-induced release **(Figure 4.6C&D)**.

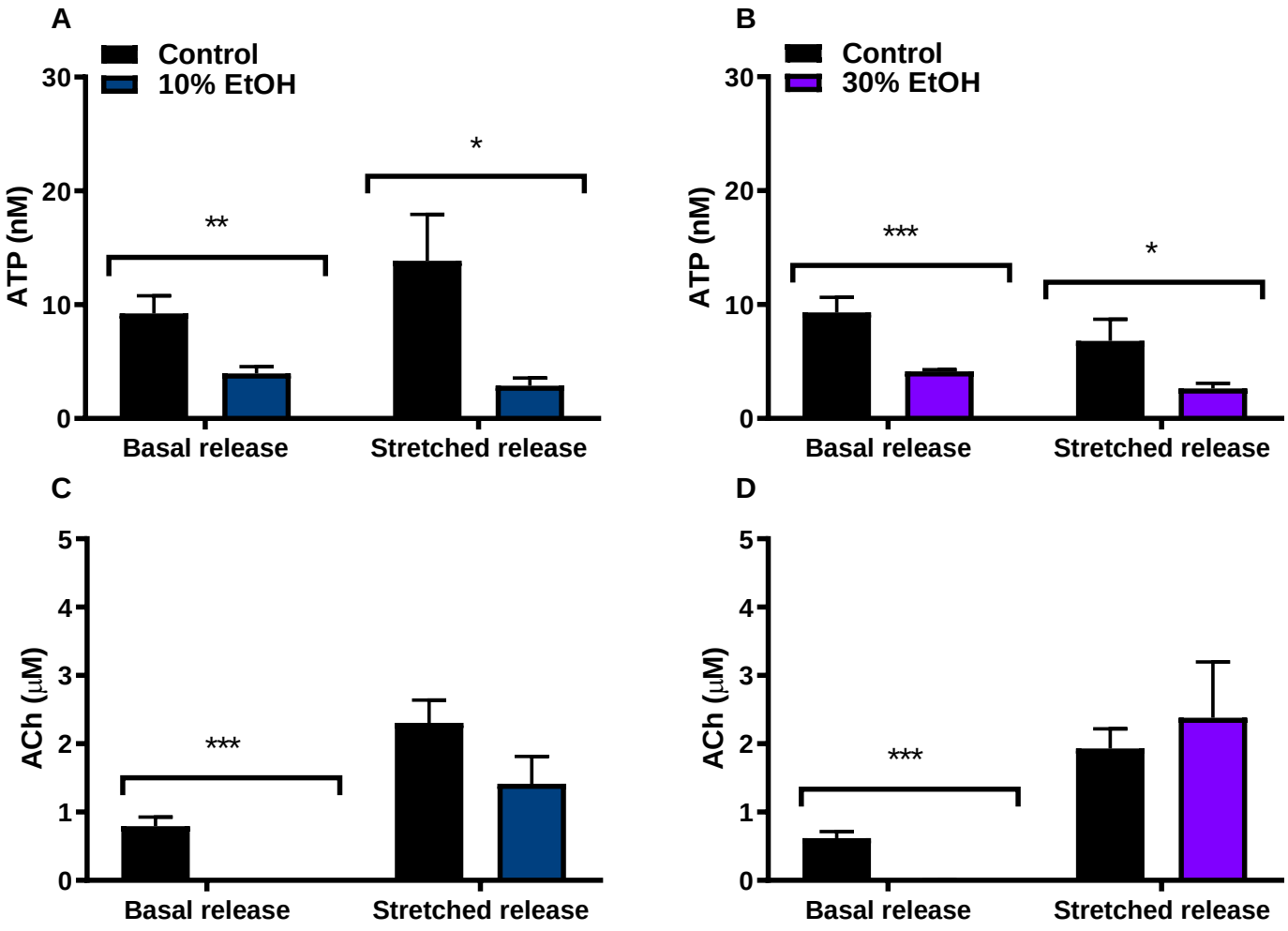

Figure 4.6: Basal and stretch induced ATP (A&B) and ACh (C&D) release from pig urothelium/lamina propria luminally pre-treated with control solution (0.9% saline), 10% EtOH (A&C) or 30% EtOH (B&D). Data is represented as mean ± SEM (n≥6) analysed by an unpaired two-tailed t-test (*P<0.05, **P<0.01, ***P<0.001 control vs. 10% or 30% EtOH).

Spontaneous contractile activity of the urothelium/lamina propria and detrusor following pre-treatment with luminal ethanol

To investigate if luminal pre-treatment with ethanol had affected the spontaneous contractile activity of the bladder, the average amplitude and frequency of spontaneous contractions in urothelium/lamina propria and detrusor strips were calculated as shown in **Figure 4.7**.

Overall, the amplitude of spontaneous contractions in all preparations of urothelium/lamina propria was significantly greater than that found in the detrusor **(Figure 4.8A&B)**. For urothelium/lamina propria tissues that were pre-treated with 10% ethanol, there was a significant increase in the amplitude of spontaneous activity (10% EtOH 6.6±1.4 mN, n=7) compared to control pre-treated tissue (control 3.2±0.5 mN, P<0.05, n=7) **(Figure 4.8A)**, with a downward trend frequency that was not quite significant **(Figure 4.8C)**. No change in amplitude or frequency occurred in the 30% ethanol experimental group when it was matched to the control treated tissues **(Figure 4.8B&D).**

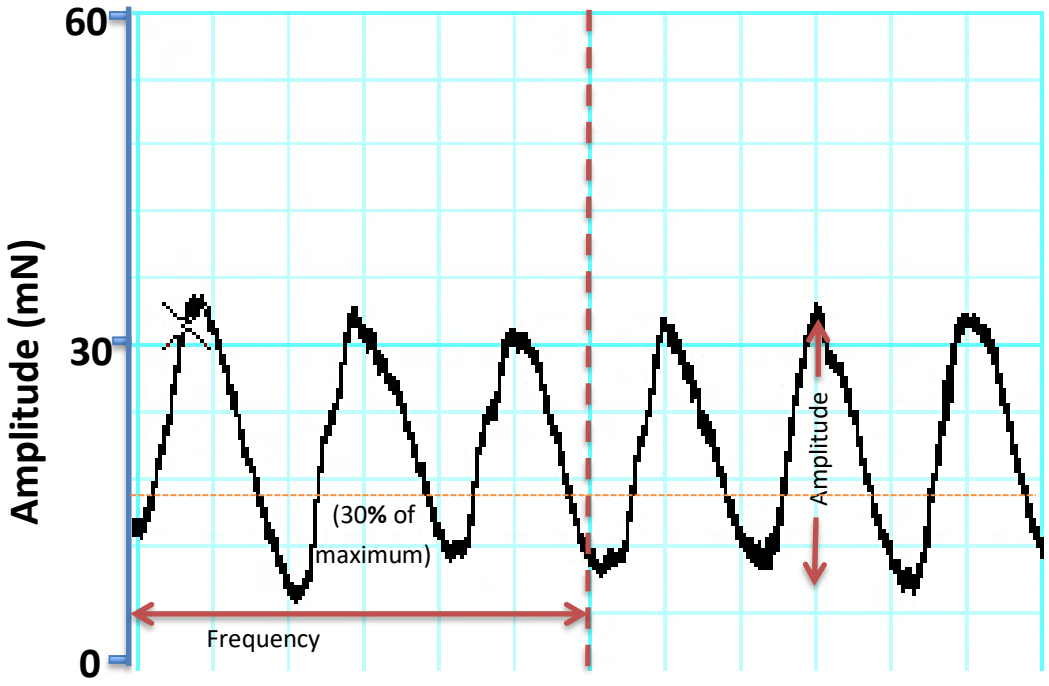

Figure 4.7: Original recording of the spontaneous activity in the untreated pig urothelium/lamina propria.

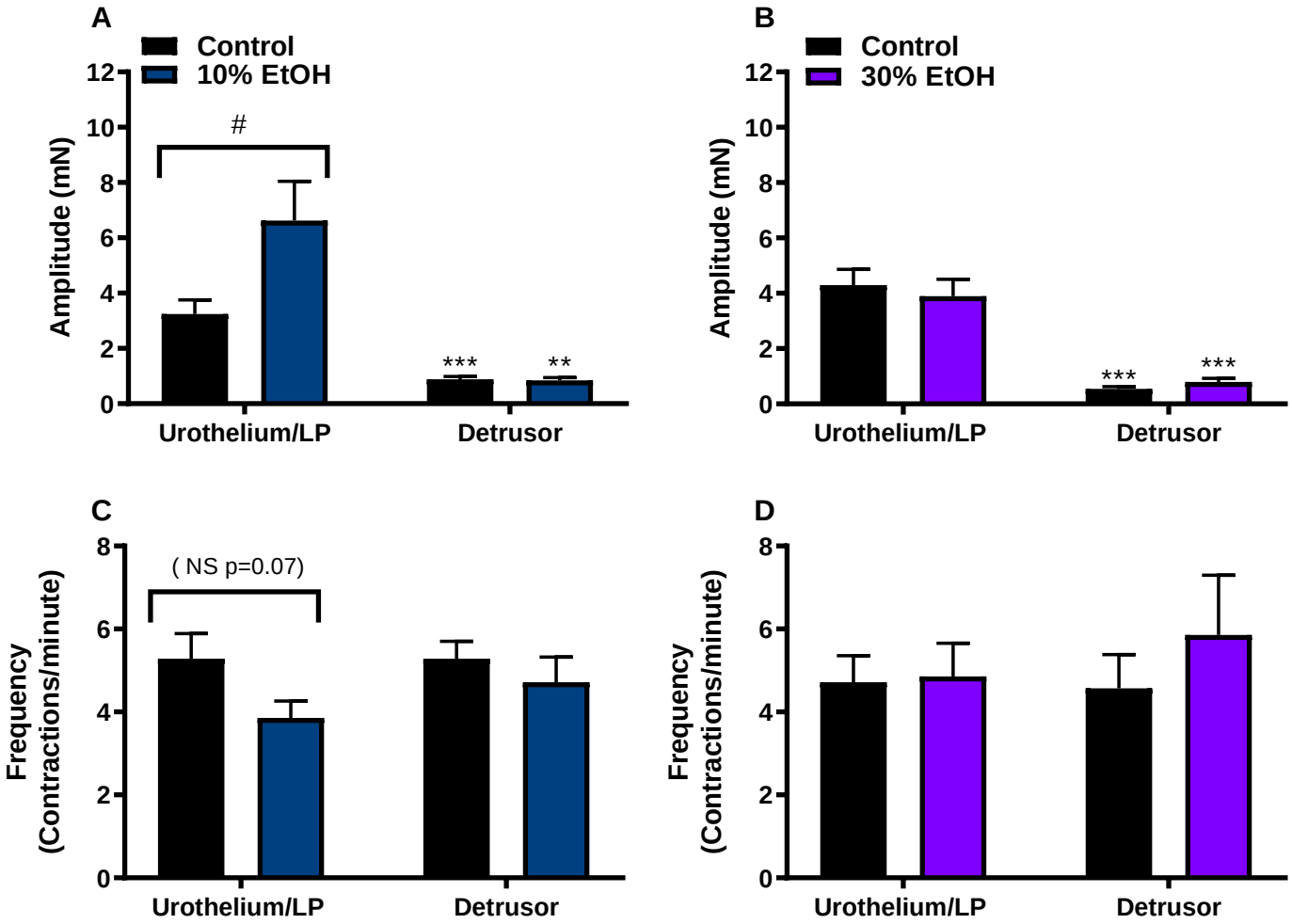

Figure 4.8: The amplitude (A&B) and frequency (C&D) of spontaneous activity in the pig urothelium/lamina propria compared to the detrusor and between matched tissue treatment groups pre-treated with control solution (0.9% saline), 10% EtOH or 30% EtOH. Data is represented as mean ± SEM (n=7) analysed by an unpaired two-tailed t-test (P<0.01, ***P<0.001 Urothelium/LP vs. Detrusor), (#P<0.05 control vs. 10% EtOH).**

Contractile response of urothelium/lamina propria, detrusor and intact tissue to ATP and KCl following ethanol pre-treatment

The contractile responses to purinergic stimulation and to non-receptor mediated stimulation were investigated using ATP (1 mM) and KCl (60 mM) (respectively) on isolated urothelium/lamina propria, detrusor and intact strips following ethanol pre-treatment.

After pre-treatment with 10% ethanol, the contractile responses of the urothelial/lamina propria, detrusor and intact strips to ATP **(Figure 4.9A,C&E)** and KCl **(Figure 4.10A,C&E)** remained much the same as control pre-treated tissues.

For urothelial/lamina propria tissues pre-treated with 30% ethanol, a significant decrease occurred in contractile sensitivity to ATP (30% EtOH 5.9±1.1 mN vs. control 10.8±1.6 mN, $P<0.05$, n=8), **(Figure 4.9B)** while the contractile response to KCl (**Figure 4.10B**) was significantly enhanced compared to control pre-treated tissues (30% EtOH 37.05±4.41 mN vs. control 21.8±3.6 mN, $P<0.05$, n=8). The response of detrusor tissues in the 30% ethanol pre-treated group to ATP **(Figure 4.9D)** tended to be reduced but this reduction did not quite reach statistical significance while the response of the same tissues to KCl (**Figure 4.10D**) remained unchanged to the matched controls. Similarly, the response of the intact tissue strips to ATP **(Figure 4.9F)** and KCl **(Figure 4.10F)** were also unaltered when compared to the control pre-treated tissues.

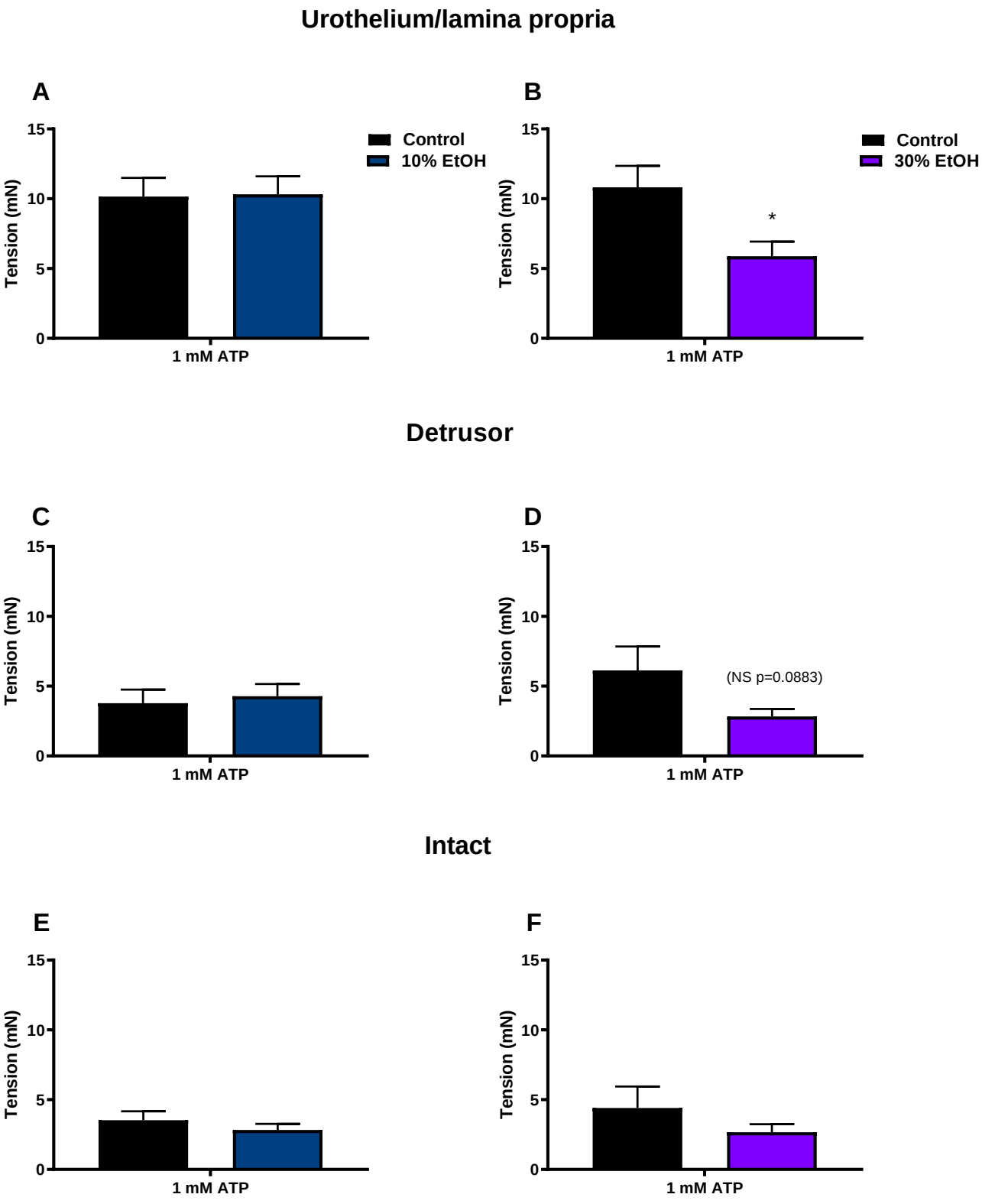

Figure 4.9: The response to 1 mM ATP from urothelium/lamina propria (A&B), detrusor (C&D) and intact bladder tissues (E&F) that had been luminally pre-treated with control solution (0.9% saline), 10% EtOH (A,C&E) or 30% EtOH (B,D&F). Data is represented as mean ± SEM (n≥7) analysed by an unpaired two-tailed t-test (*P<0.05 control vs. 10% or 30% EtOH).

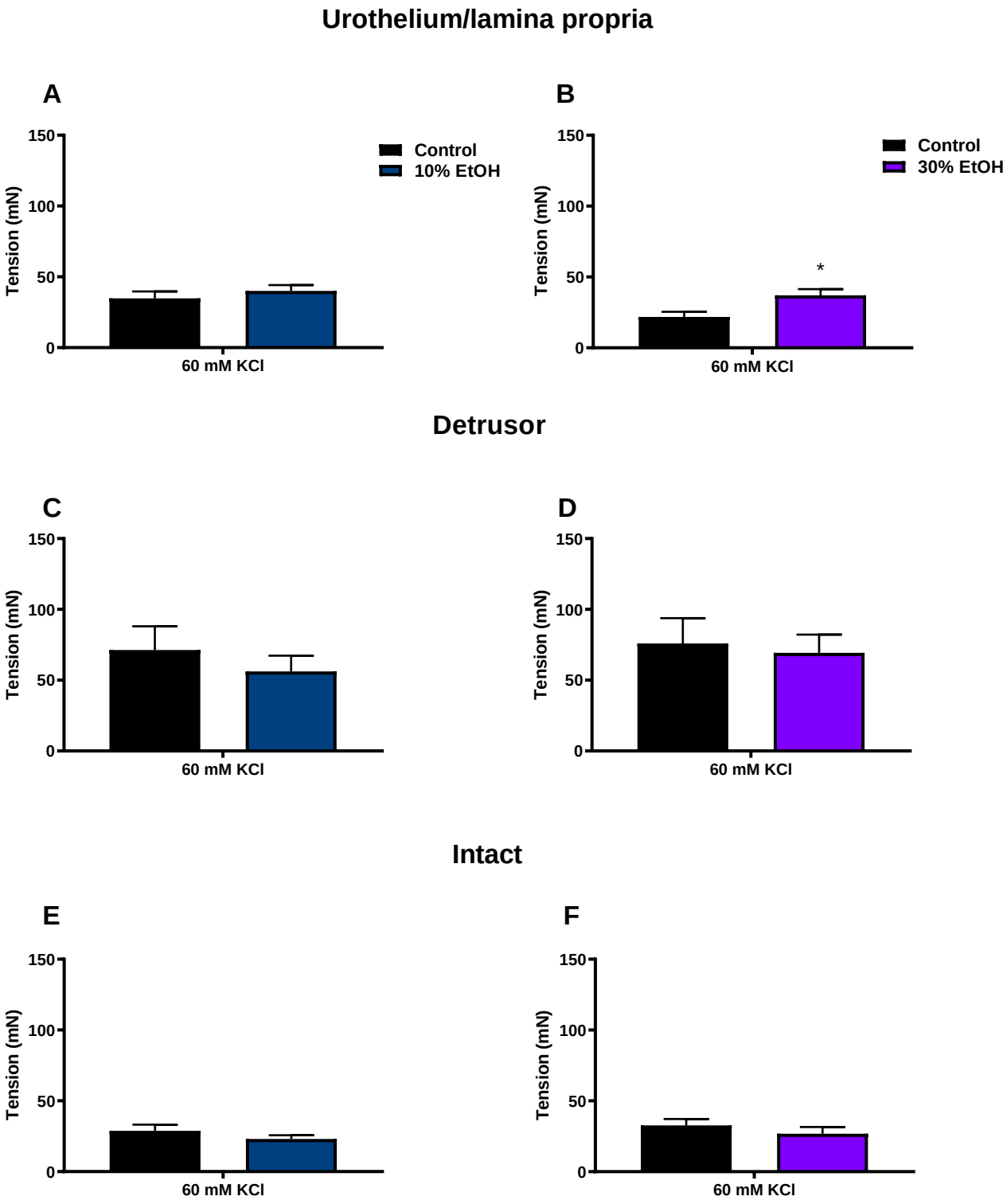

Figure 4.10: The response to 60 mM KCl from urothelium/lamina propria (A&B), detrusor (C&D) and intact bladder tissues (E&F) that had been luminally pre-treated with control solution (0.9% saline), 10% EtOH (A,C&E) or 30% EtOH (B,D&F). Data is represented as mean ± SEM (n=8) analysed by an unpaired two-tailed t-test (*P<0.05 control vs. 10% or 30% EtOH).

Contraction and relaxation responses to pharmacological agents following ethanol pre-treatment

The responses of urothelium/lamina propria, detrusor and intact strips to cumulative concentrations of carbachol were examined to determine if muscarinic receptor-mediated contractions were altered by luminal pre-treatment with ethanol. **Figure 4.11** represents the detrusor responding to cumulative concentrations of carbachol. Following a washout period, cumulative doses of the β-adrenoceptor agonist isoprenaline were used on detrusor strips pre-contracted with carbachol to assess if any changes had occurred to the relaxation responses following pre-treatment.

Cumulative concentration-response curves to carbachol were similar in the 10% ethanol and control pre-treated tissues, and neither pEC_{50} nor maximum contractile responses were affected **(Figure 4.12A,C&E, Table 4.1)**.

In contrast, pre-treatment with 30% ethanol significantly enhanced the contractile responses of the urothelium/lamina propria and detrusor to maximum carbachol stimulation by approximately 30% compared to the control treated tissues while there were no changes to the pEC_{50} values **(Figure 4.12B&D, Table 4.1)**. The contractile enhancements observed in the urothelium/lamina propria and detrusor tissues after pre-treatment with 30% ethanol were not reflected in the intact tissues which remained similar to the matched controls **(Figure 4.12F, Table 4.1)**. The presence of the urothelium/lamina propria inhibited maximum detrusor contractions to carbachol by 40% after pre-treating the tissues with 30% ethanol in contrast to 15% inhibition found in the controls.

Following pre-treatment with 10% or 30% ethanol, the isoprenaline-induced relaxation of the detrusor tissues was also unaltered when it was compared to control pre-treated tissues with no change to pEC_{50} between all pre-treated groups **(Figure 4.13A&B, Table 4.1)**.

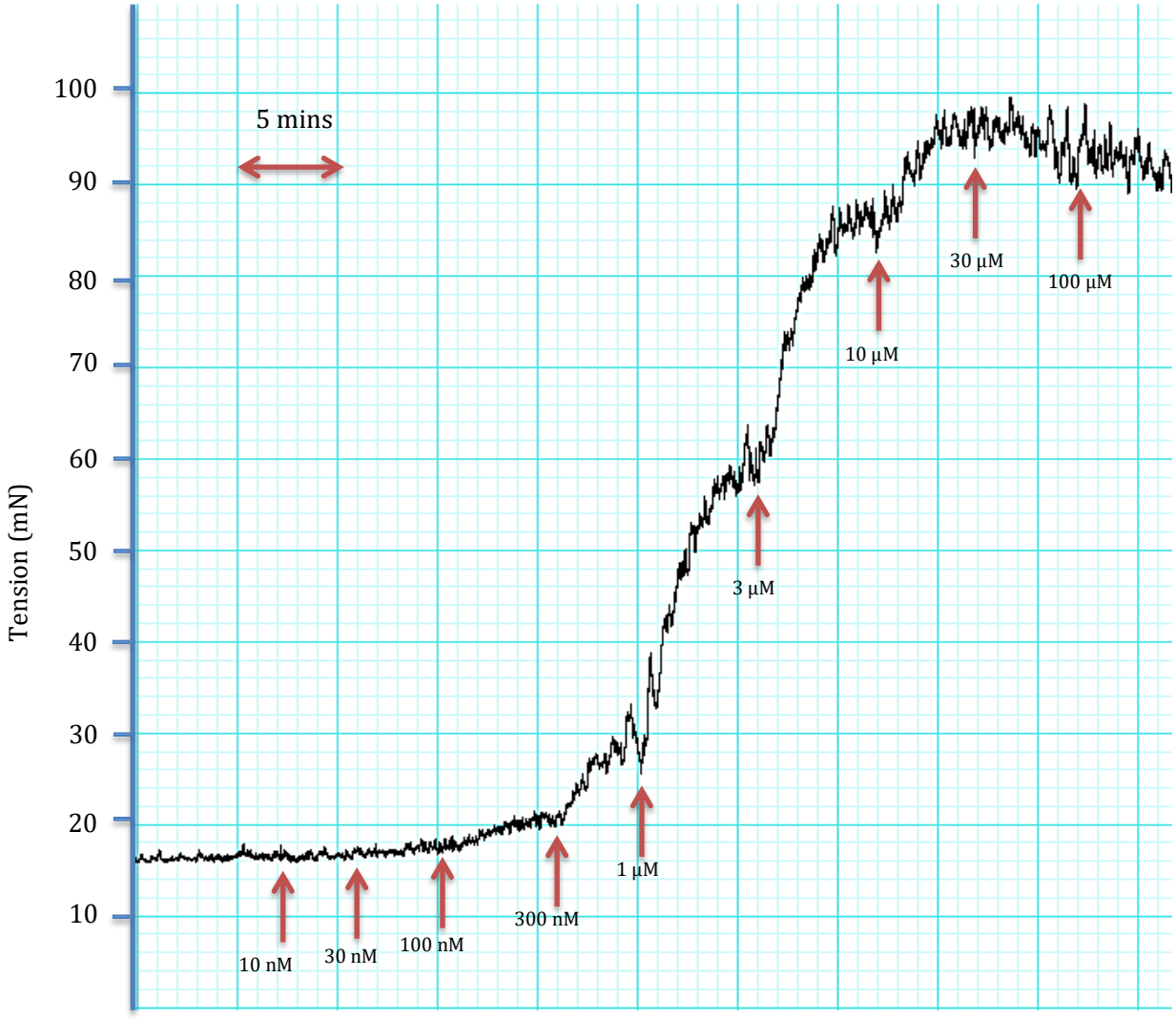

Figure 4.11: Representative trace of untreated pig detrusor tissue contracting in response to cumulative concentrations of carbachol.

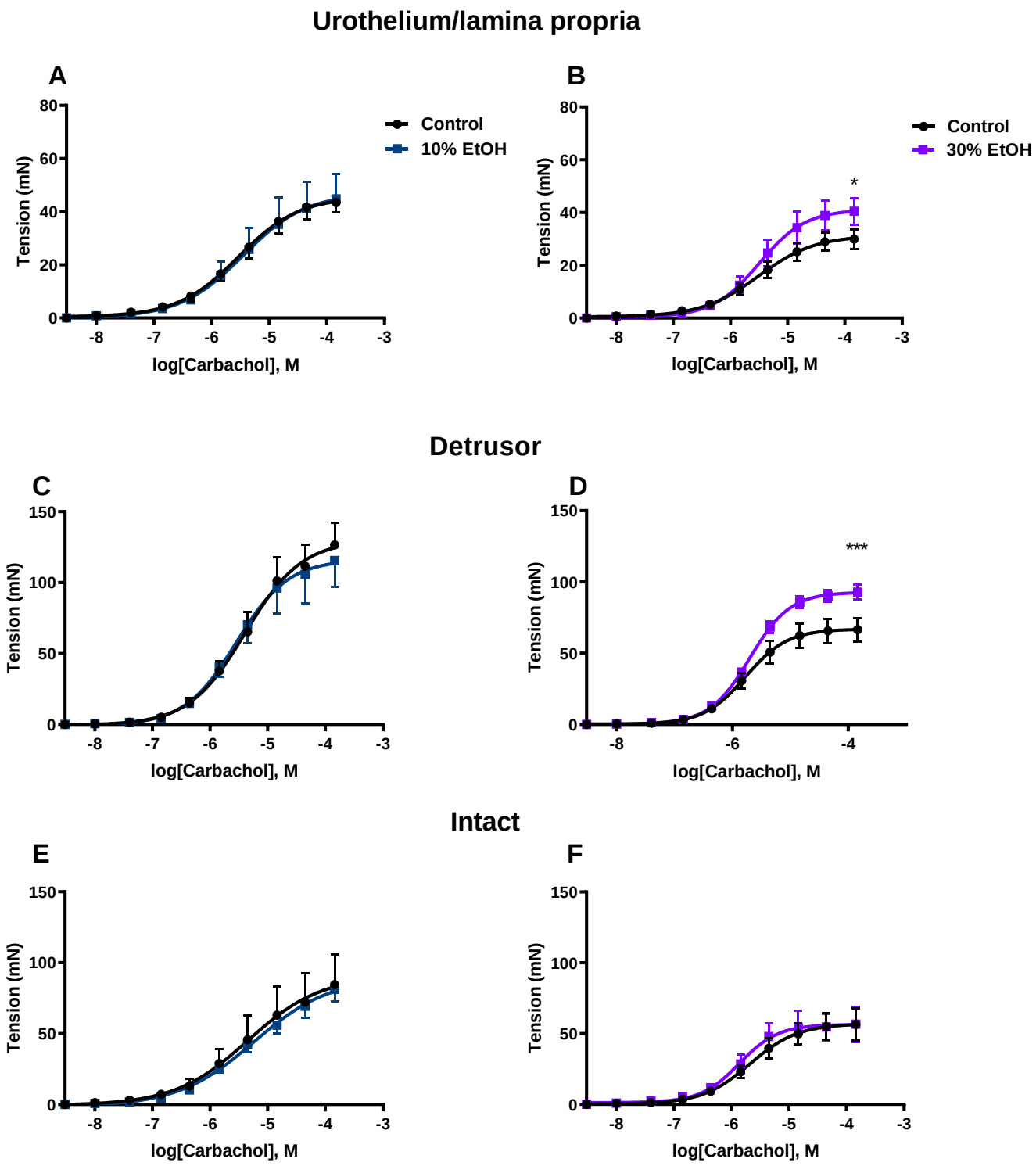

Figure 4.12: The contractile responses of pig urothelium/lamina propria (A&B), detrusor (C&D) and intact tissue (E&F) luminally pre-treated with control solution (0.9% saline), 10% EtOH (A,C,E) or 30% EtOH (B,D,F) to cumulative concentrations of carbachol. Data was analysed with multiple comparison F-test followed by an unpaired two-tailed t-test (n≥6), (*P<0.05, ***P<0.001, control vs. 10% or 30% EtOH).

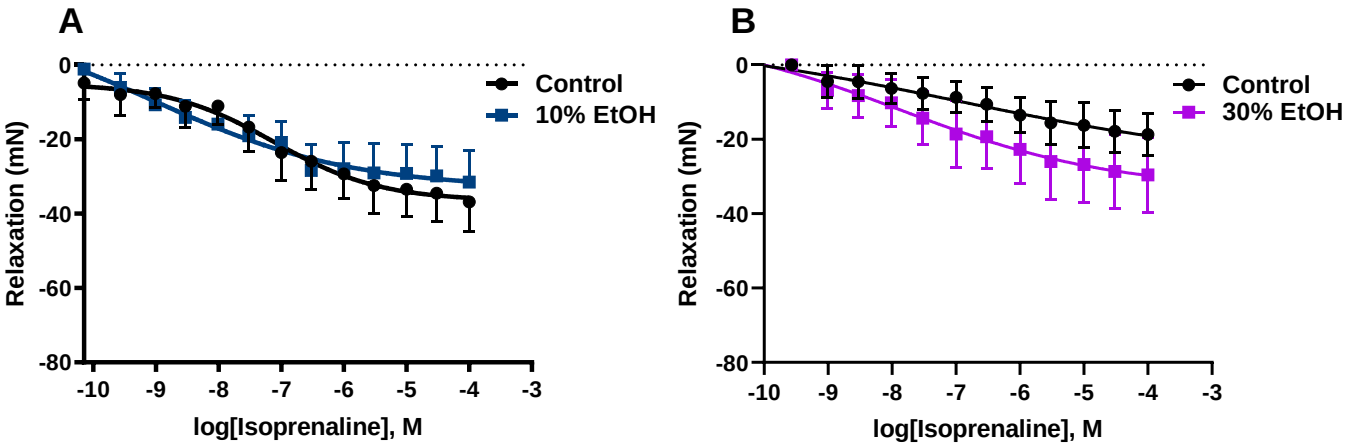

Table 4.13: The relaxation response of the detrusor following luminal pre-treatment with control solution (0.9% saline), 10% EtOH (A,C,E) or 30% EtOH (B,D,F) to cumulative concentrations of isoprenaline. Data was analysed with multiple comparison F-test followed by an unpaired two-tailed t-test (n=8).

Table 4.1: Mean±SEM maximum responses and pEC_{50} values for carbachol and isoprenaline. Data was analysed with multiple comparison F-test followed by an unpaired two tailed t-test (n≥6), (*p<0.05, ***P<0.001, control vs. 10% or 30% EtOH).

	Control (0.9% saline)	10% EtOH	Control (0.9% saline)	30% EtOH
Carbachol	**Urothelium/Lamina Propria**			
pEC_{50} (± SEM)	5.53 ± 0.1	5.45 ± 0.2	5.51 ± 0.15	5.5 ± 0.14
Maximum response (mN)	45.63 ± 3.68	46.9 ± 8.78	31.33 ± 2.74	41.14 ± 3.47 *
	Detrusor			
pEC_{50} (± SEM)	5.39 ± 0.16	5.55 ± 0.17	5.77 ± 0.11	5.7 ± 0.04
Maximum response (mN)	129.9 ± 12.96	116.1 ± 11.71	66.91 ± 4.06	92.81 ± 2.16 ***
	Intact			
pEC_{50} (± SEM)	5.35 ± 0.47	5.24 ± 0.2	5.68 ± 0.16	5.86 ± 0.17
Maximum response (mN)	90.67 ± 25.2	89.57 ± 10.92	56.99 ± 5.12	56.46 ± 4.86
Isoprenaline	**Detrusor**			
pEC_{50} (± SEM)	7. 11 ± 0.6	9.04 ± 4.13	6.44 ± 1.79	7.17 ± 1.36
Maximum relaxation (mN)	-36.51 ± 6.16	-33.23 ± 10.34	-21.86 ± 15.04	-31.85 ± 14.34

Nerve-evoked responses of the detrusor

Parasympathetic nerve activity of the detrusor was examined by EFS to determine the impact of treatment. **Figure 4.14A** demonstrates a trace of an EFS recording producing contractions in which increased frequency enhances the amplitude of detrusor contractions. This contractile activity is substantially reduced by the addition of the muscarinic antagonist, atropine (1 μM) with no further inhibition on the addition of α,β-mATP(10 μM) to desensitize the P2X receptors.

The addition of atropine significantly reduced the contractile responses of the control pre-treated tissue at 5,10 and 20 Hz stimulation by 83, 87 and 93 % respectively **(Figure 4.14B, Table 4.2)**. The addition of α,β-mATP in combination with atropine did not change the responses when stimulated at 5,10 and 20 Hz **(Figure 4.14B, Table 4.2)** indicating that ACh is the primary neurotransmitter for pig tissue.

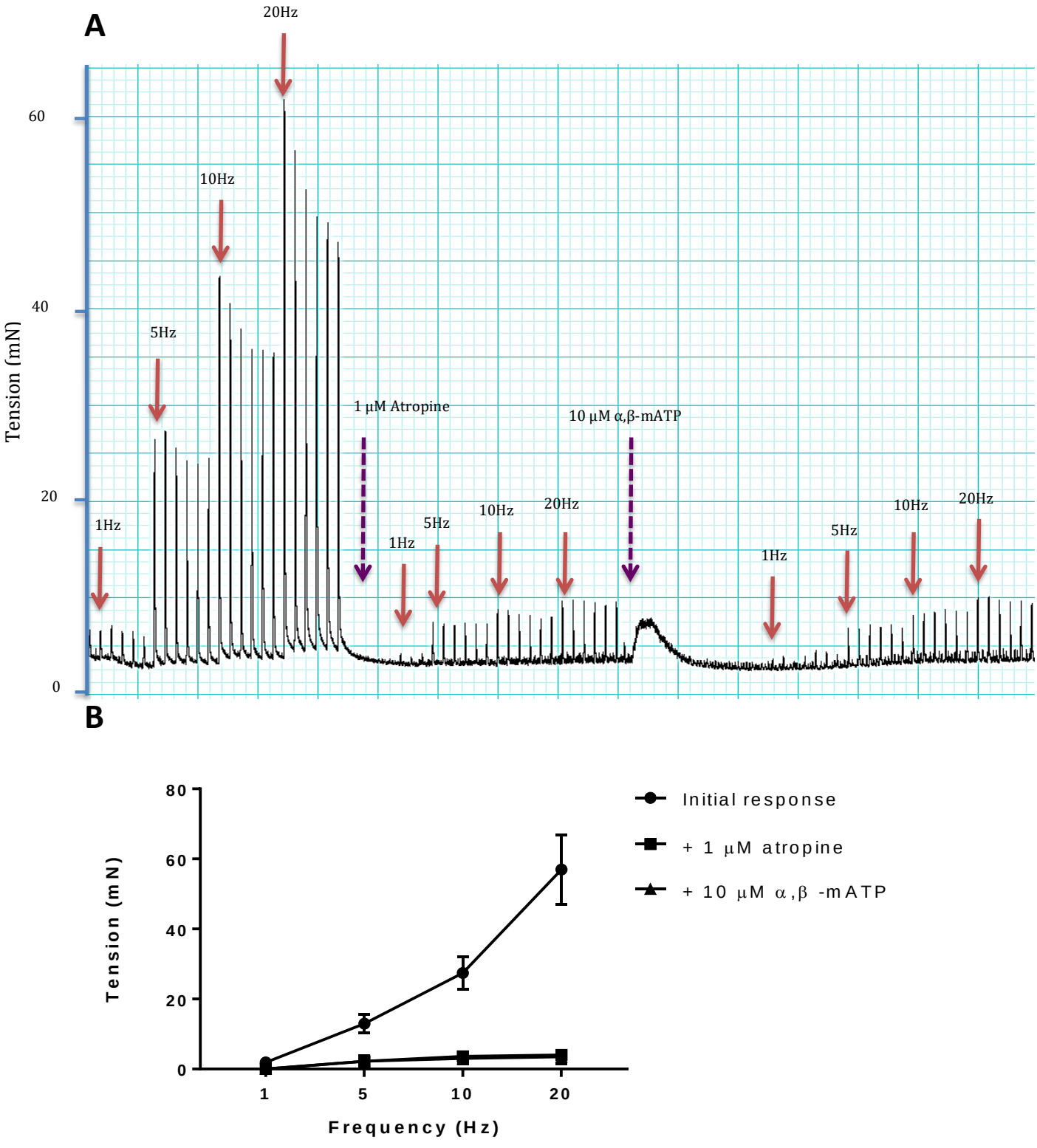

Figure 4.14: A-Representative recording of nerve-mediated contraction of the pig detrusor smooth muscle to electrical field stimulation (EFS) (20v, 1ms pulse width, 5s train) with the addition of 1 µM atropine and 10 µM α,β –mATP. B- Analysis of detrusor contraction to EFS and in the presence of 1 µM atropine and 10 µM α,β –mATP. Data is represented as mean ± SEM (n≥4).

Table 4.2: Mean±SEM initial responses to EFS at 1, 5, 10, 20 Hz in the absence and presence of 1 µM atropine and 10 µM α,β –mATP. Analysed by a paired one-tailed t-test (*P<0.05 vs. initial response)

Mean ± SEM	Initial response	+ 1 µM Atropine	+ 10 µM α,β-mATP
1 Hz	1.92 ± 0.67	0	0
5 Hz	12.95 ± 2.64	2.24 ± 0.68 *	2.27 ± 1.54*
10 Hz	27.41 ± 4.61	3.66 ± 0.76 *	3.03 ± 1.6 *
20 Hz	56.93 ± 9.99	4.06 ± 0.89 *	3.48 ± 1.88 *

Contractile responses of the detrusor to electrical field stimulation following ethanol pre-treatment

For tissues pre-treated with 10% ethanol, there was no change to nerve-evoked contractile responses of the detrusor compared to control pre-treated tissues for all frequencies tested by EFS **(Figure 4.15A)**. The addition of the muscarinic antagonist atropine produced similar reductions in responses to EFS when compared to control pre-treated tissues which remained unchanged after the purinergic receptors had been desensitized with α,β m-ATP and similar to the controls **(Figure 4.15C&E).** This indicates that ACh is the main neurotransmitter and is unaffected by pre-treatment with 10% ethanol.

For tissues pre-treated with 30% ethanol, there was a significant increase in responses to EFS at the highest frequency (20 Hz) tested (30% EtOH 105.7±19.2 mN vs. control 56.9±10 mN, P<0.05, n=7), **(Figure 4.15B)**. Electrical field stimulation in the presence of atropine reduced the responses similar to that of control pre-treated tissues while the responses after purinergic desensitization also remained unchanged and similar to the control tissues **(Figure 4.15D&F)** demonstrating that ACh is also the dominant neurotransmitter after pre-treatment with 30% ethanol.

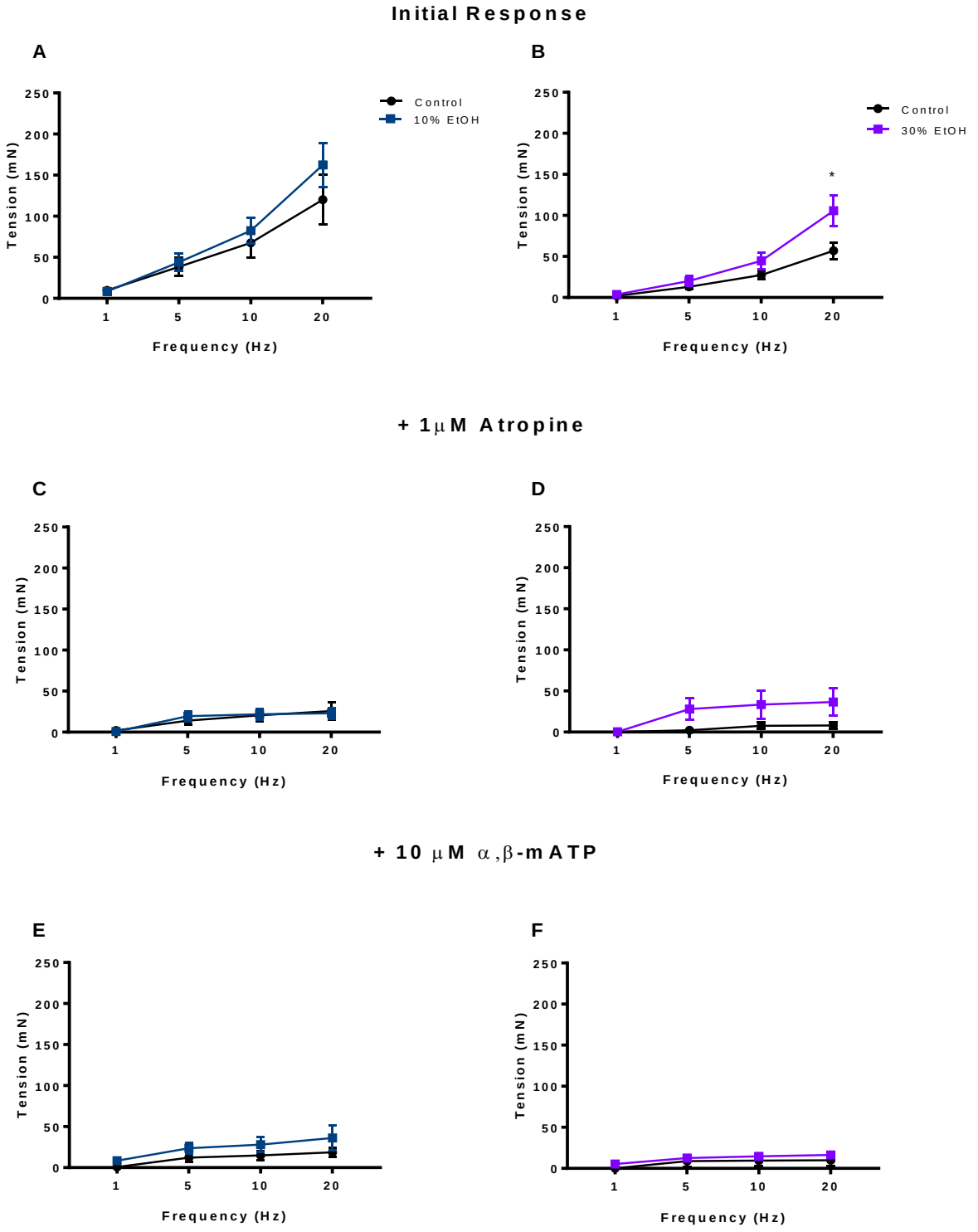

Figure 4.15: Responses to EFS (20v, 1ms pulse width, 5s train) of pig detrusor tissue luminally pre-treated with control solution (0.9% saline), 10% EtOH (A,C&E) or 30% EtOH (B,D&F) and in the presence of 1 μM atropine (C&D) and 10 μM α,β –mATP (E&F). Data is represented as mean ± SEM (n=7) analysed by an unpaired two-tailed t-test (*P<0.05, control vs. 10% or 30% EtOH).

4.5 Discussion

Intravesical treatment of the bladder involves direct contact of the chosen compound with the urothelium of the bladder. However, the interactions of ethanol with biological membranes are complex, as it is known to interact with plasma membranes and can readily diffuse intracellularly and interact with the cellular organelles (Sun and Sun, 1985). One review on the biological effects of ethanol has suggested that although the membrane hypothesis can account for some of the biochemical effects, there is increasing evidence for it having interactions with G-proteins (Hoffman and Tabakoff, 1990).

Ethanol is a small polar molecule that is both hydrophilic and lipophilic. Its lipophilic qualities (despite being quite low) would allow it to diffuse across plasma membranes and the hydrophilic properties make it completely soluble in water (Kent, 2012, Fernandes et al., 2017).

Ethanol has a molecular weight of 46.07, and generally, the molecular weight is related to molecular size. The diameter of protein channels in the cell membrane are approximately 10A which is equivalent to a molecular weight of 200, and intercellular gap junctions are approximately 15A. Accordingly, non-ionized substances with a molecular weight of less than 200 are thought to permeate the cell membrane via its protein ion channels while substances with a larger molecular weight would pass through the intercellular gap junctions. This would suggest that ethanol could easily diffuse across cellular membranes and through the intercellular spaces (Mishina et al., 1986, The Essential Chemical Industry online, 2016.).

Regarding the urothelial GAG layer of the bladder, the components that make up the proteoglycan layer consist of heparan sulphate, chondroitin sulphate, dermatan sulphate and hyaluronic acid. These are negatively charged and bound to a core protein that also has a negative charge except for hyaluronic acid which is neither bound nor sulphated (Janssen et al., 2013). With this in mind, ethanol applied to the luminal surface would most likely have concentration-dependent effects as the two negatively charged compounds (ethanol and GAG layer) would initially repel, yet the lipophilic properties would allow diffusion across plasma membranes and into the intercellular spaces while higher concentrations would allow for greater diffusion (Sun and Sun, 1985, Kwak et al., 2012).

It is unclear how far ethanol would diffuse from the lumen throughout the layers of the bladder as directly beneath the GAG layer are the umbrella cells with their asymmetric unit membrane, uroplakins, tight junctions and adherence proteins which may slow down the penetration

process (Apodaca, 2004, Janssen et al., 2013). If ethanol can penetrate past the urothelium/lamina propria, there is also a capillary network in the submucosa that protects the underlying detrusor from harmful substances (Mishina et al., 1986).

The effect of luminal ethanol on the urothelium/lamina propria during and post-treatment

During treatment with 10% and 30% ethanol, ATP release was significantly increased by four-fold and 185-fold respectively. The P2X3 and P2X2/3 receptors found on the urothelium/lamina propria and afferent nerves just below, are believed to be sensory targets for ATP, playing a role in sensory functions including pain (Ferguson et al., 1997, O'Reilly et al., 2002, Tempest et al., 2004, Kumar et al., 2007, Birder, 2010, Ford and Cockayne, 2011, Burnstock, 2014, Svennersten et al., 2015). Some studies have reported that ATP delivered onto the skin produces pain that is mediated by the C-fibres and concentrations of >30 nM are sufficient to cause wealing in human skin (Coutts et al., 1981, Hamilton et al., 2000). In the present study, the concentrations of ATP release during treatment with 10% or 30% ethanol were approximately 16 nM and 1.7 µM respectively and indicates that during treatment, 10% ethanol may cause some mild irritation while this may be considerably intensified during treatment with 30% ethanol.

Generally, ATP is broken down by membrane-bound ecto-nucleotidases that convert it to adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenosine (Burnstock, 2011). In zebrafish brain membranes, acute ethanol exposure for 1-hour at concentrations varying from 0.25-1% inhibited ecto-ATPase activity significantly decreasing ATP hydrolysis, which could explain the enhancement of ATP concentrations found in this study (Rico et al., 2008). However, the most likely explanation for the increases in release is dispersement of ATP due to cellular damage by ethanol which in other tissues has been found to be concentration-dependent (Tapani et al., 1996, Neuman et al., 2002, Lewis and Lewis, 2006).

Acetylcholine release remained unchanged for tissues while being treated with 10% ethanol. However, release was significantly reduced ten-fold for tissues that were treated with 30% ethanol. As the majority of non-neuronal ACh release is suspected to come from the urothelium, the significant decrease during treatment could reflect the state of the urothelium and suggests that thinning and denudation has occurred during treatment (Yoshida et al., 2006). In support, this has been reported in a histological analysis of rat bladders after intravesical

exposure to 30% ethanol (Byrne et al., 1998, Yoshida et al., 2006). Also, the activity of LDH was found to be significantly higher after both ethanolic treatments suggesting that these concentrations of ethanol were sufficient to overcome the GAG layer and have interacted with the superficial cells of the bladder.

Histological analysis of bladder tissues following pre-treatment with 10% ethanol revealed no change to urothelial thickness. However, a compromised urothelium/lamina propria with a 75% decrease in urothelial thickness was clearly visible after pre-treatment with 30% ethanol. Although there was no observable change to bladder tissues pre-treated with 10% ethanol, one study with liver cell lines has shown this concentration of ethanol to be cytotoxic after a 50-60-minute exposure (Tapani et al., 1996). Urothelial tissues in the present study were exposed for 30-minutes with an intact GAG layer as well as umbrella cells and tight junctions so less cytotoxic activity would be expected.

The urothelium/lamina propria function following ethanol pre-treatment

With these results in mind, it was important to determine if urothelial/lamina propria behaviour had been impaired by treatment. Urothelial stretch results in the release of ATP and ACh which are believed to be indicators of bladder fullness modulators of bladder activity (Kumar et al., 2004, Yoshida et al., 2006, Daly et al., 2010).These next experiments examined the release of ATP and ACh under basal and stretch conditions.

ATP

As stretch of the urothelium evokes the release of ATP, the control tissues in both ethanolic groups under stretch conditions released ATP that was similar to basal releases. Kushida and Fry (2016) demonstrated that ATP release from the guinea pig urothelium in basal conditions is cyclical and is associated with spontaneous urothelial contractions. Sui et al. (2014) also identified significant ATP surges in urothelial sheets of human and in guinea pig bladders. For this reason, the lack of change in ATP release in stretch conditions compared to basal release found in the control pre-treated tissues could be due to cyclical variation and demonstrates the difficulty interpreting the results for urothelial based ATP release.

Regardless of the results from the control tissues, there was a 58% and 80% decline in basal ATP release found in tissues that had been pre-treated with 10% and 30% ethanol respectively. Similarly, stretch evoked ATP release was also reduced by 80% and 60% for the 10% and 30% ethanol groups respectively.

The decreases found in basal ATP release for both ethanol groups could be due to cyclical variations as discussed earlier, or it could also be due to basal ACh release that was below detection for both treated samples. It has previously been identified that muscarinic agonist on human and guinea pig urothelial tissue elicits ATP release (Sui et al., 2014, McLatchie et al., 2014). Moreover, it has been proposed that urothelial ATP enhances its own release, and it has been found that stimulation of P2Y receptors, which are also G-protein coupled receptors, elicits this release in guinea pig and human urothelial preparations and also in rat urothelial cells (Chopra et al., 2008, Sui et al., 2014). Furthermore, approximately >70% of ATP originates from the upper uroepithelial cells in guinea pig bladders (Sui et al., 2014). Consequently, the lack of basal ACh release in both ethanol pre-treated samples for this study could be influencing basal ATP release as well as the lack of fully functional urothelium in the tissues pre-treated with 30% ethanol. Ethanol may have also perturbed the function of P2Y receptors in tissues pre-treated with 10% ethanol while pre-treatment with 30% ethanol may have reduced the P2Y population additionally contributing to the reductions found in basal ATP release.

It is unclear what would be contributing to the reduction in stretch-induced ATP for tissues pre-treated with 10% or 30% ethanol. However, many factors could be involved including the depletion of Ca^{2+} stores as a result of the elevated ATP releases found during ethanol treatment (Trevisani et al., 2002, Matsumoto-Miyai et al., 2011). Also, acute ethanol exposure on cells and tissues has been found to be a potent activator of adenylate cyclase activity and 3'-5'-cyclic adenosine monophosphate (cAMP) (Hoffman and Tabakoff, 1990). As mentioned earlier, activation of M2 receptors (G_i) is thought to oppose sympathetically mediated cAMP accumulation. (Caulfield, 1993, Hegde and Eglen, 1999, Andersson, 2004) It was observed by Young et al. (2012) that methoctramine (M2 antagonist) inhibited stretch evoked ATP release from guinea pig mucosal strips. An M2 antagonist theoretically would not affect cAMP levels within the cell leaving them elevated, and this receptor has been almost exclusively found on the umbrella cells of the human urothelium (Bschleipfer et al., 2007). For tissues pre-treated with 10% ethanol, the umbrella cells with the large population of M2 receptors may have been

damaged. For tissues that had been pre-treated with 30% ethanol, the umbrella/urothelial layer is no longer intact, and the remaining M2 receptors may also be damaged leading to a significant decline of ATP release in stretched conditions.

Another explanation for the reduction in stretch-induced ATP after pre-treatment with 30% ethanol could also involve the urothelial TRPV1 channel which may no longer be present. Immunoreactivity for this receptor has been observed throughout the urothelial cell layers in the rat and human bladder. (Birder et al., 2001, Lazzeri et al., 2004b). Furthermore, in the TRPV1 knock out models, cultured mouse urothelial cells release less ATP when stimulated with a hypotonic solution while the whole mouse bladder produces less distension induced ATP (Birder et al., 2002, Grundy et al., 2018b).

Additionally, increased levels of adenosine by various cells into the extracellular space and subsequent activation of adenosine receptors has been reported after acute ethanol exposure in varying concentrations (Nagy et al., 1989, Clark and Dar, 1989, Nagy et al., 1990, Nagy, 1994). Adenosine receptor subtypes A_1 and A_3 preferably interact with G_i and A_{2a} and A_{2b} couple with G_s, stimulating cAMP (Ralevic and Burnstock, 1998). As all four adenosine receptors have been found on the urothelium, an ethanol-induced increase of adenosine, possibly by an enhanced breakdown of ATP, could be interfering with stretch-induced ATP via the urothelial adenosine receptors (Yu et al., 2006). A study by Dunning-Davies et al. (2013) identified that in the presence of 1-2 µM adenosine, the urothelial stretch release of ATP in the rabbit bladder was significantly attenuated and was completely abolished by 10 µM of adenosine. The same study by Dunning-Davies et al. (2013) found that antagonism of P2X3 and P2X2/3 receptors also diminished distension induced ATP release. As ethanol also reportedly interferes with P2X receptors on the plasma membrane modulating ion flow, ethanol interference of P2X3 and P2X2/3 as well as its influence on adenosine levels interacting with adenosine could all be factors contributing to the reduction in stretch-based ATP release (Wise, 2017).

ACh

Under basal conditions, the release of ACh from tissues pre-treated with 10% or 30% ethanol was below detection. It has been reported in whole rat bladder preparations and in cultured rat urothelial cells that the urothelial application of ATP evokes ACh release in basal conditions (Hanna-Mitchell et al., 2007, Stenqvist et al., 2017). For tissues pre-treated with 10% ethanol, the low levels of ATP may in-turn be influencing basal ACh conditions. Also, lowered ethanol

concentrations <200 mM have been found to enhance acetylcholinesterase activity which may be a contributing factor for the reduced levels found in these tissues (Shin et al., 1991). However, for tissues treated with 30% ethanol, the diminished release is most likely due to an eroded urothelium as it has been observed in human bladder samples that the majority of choline acetyltransferase (synthesizes ACh) is found in the urothelial cells (Yoshida et al., 2006). While the urothelial cells in tissues remain intact for tissues treated with 10% ethanol, there also remains a possibility that this synthesizing enzyme may have also been affected by ethanol.

For the control tissues in both ethanol groups, the stretch-evoked release of ACh was significantly enhanced compared to basal conditions while pre-treatment with 10% or 30% ethanol did not alter the enhancement in stretch-induced ACh release. It has been observed that most of the ACh released by stretch in human bladder strips was significantly attenuated when the urothelium/lamina propria was removed (Yoshida et al., 2006). These results suggest that the origin of stretch released ACh is from the underlying sub-urothelial lamina/propria considering that the urothelium has almost been entirely sloughed off by 30% ethanol. Choline acetyltransferase has been identified in sub-urothelium/lamina propria albeit in much smaller quantities than the urothelial layer with sheer stress suggested as one of the releasing mechanisms for stretch based release of ACh (Yoshida et al., 2006).

The effect of ethanol on bladder contractile/relaxation mechanisms following pre-treatment

Based on the previous observations, it appears that both concentrations of ethanol have impacted the function of the urothelium/lamina propria. To determine if ethanol has diffused into the deeper layers of the bladder, the contractile function of urothelium/lamina propria, detrusor and intact strips were assessed. Although contraction of the detrusor is responsible for the expulsion of urine, the urothelium/lamina propria has been found to have contractile properties that are distinct to the detrusor. The contractile properties found in the urothelium/lamina propria are thought to be perpetuated by the sub-urothelial interstitial cells, the extensive network of blood vessels and the presence of muscularis mucosae which are irregularly arranged bundles of smooth muscle that lie midway between the detrusor and the urothelium (Dixon and Gosling, 1983, Sadananda et al., 2008, Fry and Vahabi, 2016).

The urothelium/lamina propria will contract in response to carbachol mediated by the M3 receptors as well as ATP and its breakdown products which are P2X and P2Y receptor agonists (Moro et al., 2011, Kushida and Fry, 2016).

Spontaneous activity

During the storage phase of the micturition cycle, spontaneous contractile activity of the bladder (not large enough to cause micturition) can occur *in vivo,* in isolated bladder preparations and tissue strips (Drake et al., 2005, Akino et al., 2008, Parsons et al., 2012). Spontaneous activity is suggested to be myogenic, and its origin is not clearly understood although the urothelium/lamina propria, muscularis mucosae and interstitial cells have been implicated (Levin et al., 1986, Heppner et al., 2011, Drake et al., 2017). The pig bladder urothelium/lamina propria, detrusor and intact strips are no exception to spontaneous activity (Akino et al., 2008, Moro et al., 2011). In the pig bladder, the presence of urothelium/lamina propria inhibited the effects of cromakalim (K_{ATP} channel opener) in suppressing spontaneous contractions in the detrusor suggesting it has a considerable influence on overall spontaneous activity (Akino et al., 2008). Moreover, spontaneous detrusor activity has shown to be enhanced by the urothelium/lamina propria in intact preparations of guinea pig bladder (Kushida and Fry, 2016).

For this study, the detrusor displayed smaller spontaneous contractile amplitudes when compared to the urothelium/lamina propria for all experimental groups. Interestingly, the amplitude of spontaneous contraction in the urothelium/lamina propria was enhanced by 100% after pre-treatment with 10% ethanol while there were no alterations to contractile amplitude after pre-treatment with 30% ethanol. There were also no changes to the frequency of spontaneous activity after pre-treatment with 10% or 30% ethanol.

It's believed that mediators expressed by the urothelium may have some influence over spontaneous activity such as ATP, ACh and prostaglandins, which have all been found to regulate each other to some degree in complex interactions (Maggi, 1992, Moro et al., 2011, Nile and Gillespie, 2012, Kobayter et al., 2012, Kushida and Fry, 2016). However, for these observations, it seems unlikely that urothelially released ACh or ATP are contributing substantially to spontaneous activity as both of these mediators are significantly reduced in basal conditions in ethanol pre-treated urothelial/lamina propria tissues**.** This finding is supported by Heppner et al. (2011) who found that blocking and muscarinic receptors,

desensitizing the P2X receptors and blocking neural transmission in the guinea pig urothelium/lamina propria had no bearing on spontaneous activity.

This leaves us with prostaglandins. Ethanol on skin cell preparations has been found to enhance pro-inflammatory cytokines including TNF-α and the stimuli for prostaglandins in the bladder are mediators of inflammation and urothelial injury (Maggi, 1992, Neuman et al., 2002, Shioyama et al., 2008). Moreover, prostaglandins have been found to be enhanced in various tissues in the presence of ethanol (Collier et al., 1975, Greenberg et al., 1993). To relate this to the findings of this study, in the guinea pig urothelium, COX 1 is found in the intermediate and basal layers of the urothelium and prostaglandin receptor EP1 is found through-out the urothelial layers while EP2 receptors are located in the umbrella cells (de Jongh et al., 2009, Rahnama'i et al., 2010). Prostaglandin EP1- EP4 receptors are G-protein coupled receptors. The EP2 and EP4 receptors generally mediate increases to cAMP while the EP1 receptor is associated with a contractile response. The EP3 receptor is an inhibitory receptor which mediates decreases in cAMP (Ushikubi et al., 1995). As the umbrella cells are the first cells in these tissue preparations to come into contact with ethanol, it may have impaired the function of these receptors particularly EP2 found in the umbrella cells which mediates relaxation allowing a stronger spontaneous contraction to occur in tissues pre-treated with 10% ethanol. Ethanol may also be enhancing prostaglandin production by interaction with COX 1 in the intermediate and basal cells of the urothelium as the enhancement in amplitude found in tissues treated with 10% ethanol was absent from tissues treated with 30% ethanol with an eroded urothelium.

The contractile responses to ATP and KCl, muscarinic and parasympathetic stimulation (EFS)

10% ethanol

For tissues that were pre-treated with 10% ethanol, the urothelium/lamina propria, detrusor and intact contractile responses to purinergic stimulation remained unaffected by treatment. This suggests that ethanol at this concentration has not interfered with purinergic signalling and seems less likely that 10% ethanol has interfered with P2X receptors throughout the bladder.

In the bladder, potassium chloride depolarizes the smooth muscle cell membrane and opens voltage-dependent Ca^{2+} channels resulting in the activation of the contractile machinery (Karaki et al., 1984, Heppner et al., 2011). Pre-treatment with 10% ethanol did not show any

interference to non-receptor mediated contraction for all tissues examined. Likewise, the contractile responses of the urothelium/lamina propria, detrusor and intact tissue to muscarinic receptor stimulation also remained unchanged by treatment.

Accordingly, as purinergic and muscarinic responses of the detrusor remained unchanged by pre-treatment with 10% ethanol, EFS produced no additional changes beyond the control for all frequencies tested. The contributions made by ACh and ATP to nerve-mediated stimulation also remained unchanged by pre-treatment with 10% ethanol as blockade of muscarinic receptors reduced the majority of the response to EFS which was not altered any further by desensitizing the P2X receptors. Overall, these findings demonstrate that pre-treatment with 10% ethanol does not interfere with non-receptor mediated contractile activity or nerve-mediated cholinergic and purinergic responses.

30% ethanol

In contrast to pre-treatment with 10% ethanol, purinergic stimulation following pre-treatment with 30% ethanol depressed the response of the urothelium/lamina propria by approximately 45% while the responses of the underlying detrusor or intact tissues were unaffected. In the pig bladder, smooth muscle actin staining has been found in interstitial cells that lie directly beneath the urothelium, blood vessels and weakly in the connective tissue which has been suggested to mediate the contractile activity of this particular tissue (Sadananda et al., 2008). In humans, sub-urothelial interstitial cells have been found to express P2X1, P2X2 and P2X3 receptors that have generated Ca^{2+} transients post stimulation by ATP (Cheng et al., 2011a). The smaller contractile amplitude produced by ATP in urothelial/lamina propria tissues pre-treated with 30% ethanol suggests due to the lack of urothelial cells, the purinergic signal was not amplified by the P2X or P2Y receptors to the underlying layers.

In response to KCl, the urothelium/lamina propria had an enhanced contractile response of approximately 76% while the detrusor and intact tissue responses remained unaltered**.** In the bladder, K^+ is considered to be a major urinary toxin. It has been found to enhance pain sensations and urinary urgency in patients with a compromised urothelium and is thought to act by depolarizing sensory nerves and smooth muscle (Parsons et al., 1998, Parsons, 2003). Following pre-treatment with 30% ethanol, the impaired urothelium would allow KCl to diffuse unhindered to the contractile tissues.

Moreover, muscarinic receptor stimulation enhanced the contractile response of the

urothelium/lamina propria and detrusor by approximately 35% and 30% respectively. Interestingly, the enhancement that was found in the urothelium/lamina propria and detrusor preparations did not influence the intact preparations which were unchanged to control treated tissues. As the detrusor responses to muscarinic stimulation were enhanced, it was no surprise that the contractile response of the detrusor to EFS was enhanced by 85% (20 Hz), although, antagonizing the muscarinic receptors reduced the majority of the response to EFS similar to control values which were not altered any further by desensitizing the P2X receptors.

It is difficult to ascertain the concentration of ethanol that remains behind in these tissues after luminal treatment. However, Etessami (1972) observed that in neuromuscular phrenic diaphragm preparations from the rat, ethanol in lower concentrations (<2%) increases excitability, conductivity and amplitude of contractions and that higher doses produce a blockade (2-4%). Moreover, in cultured guinea pig detrusor smooth muscle cells it was found that ethanol at concentrations of >0.3% had inhibitory effects on the L-type Ca^{2+} channels contributing to a relaxation response (Malysz et al., 2014). This indicates that the concentration of ethanol that remains in the detrusor is <0.3%. Ethanol in concentrations of 0.1-3% does not appear to be neurotoxic which is consistent with earlier reports that 5% ethanol on hepatocytes was non-cytotoxic (Tapani et al., 1996, Trevisani et al., 2002). However, ethanol in non-toxic concentrations, on various sensory tissues in the rat, has been found to activate TRPV1 channels on sensory afferent neurons by releasing SP (Trevisani et al., 2002). The release of neuropeptides such as SP and CGRP (in the human, NKA is also usually expressed with SP and CGRP) has been associated with neurogenic inflammation (Maggi, 1990, Smet et al., 1997) Moreover, NKA and SP in the bladder has been positively correlated to promote contractile activity in the bladder (Maggi et al., 1984, Santicioli et al., 1986, Persson et al., 1991, Templeman et al., 2003). Maggi et al. (1984) found that SP enhanced the amplitude of TTX sensitive distension induced contractions in the rat bladder. Ethanol has also been found to cause a concentration-dependent increase in intracellular Ca^{2+} in human TRPV1 expressing HEK293 cells compared to wild-type HEK293 cells with no enhancement of the carbachol (muscarinic agonist) Ca^{2+} response in the same cells (Trevisani et al., 2002).

The evidence mentioned above suggests that the enhancement to muscarinic stimulation found in the urothelium/lamina propria that had been pre-treated with 30% ethanol could reflect a lack of urothelial cells. This would allow for easier passage of ethanol to interact and activate TRPV1 channels which are found abundantly in the sub-urothelial layer (Lazzeri et al., 2004b).

Moreover, with much of this cell layer stripped away, the contractile components of the urothelium/lamina propria would be more sensitive to irritative substances (including KCl). It seems a little less likely for carbachol-induced prostaglandins to be playing a major role in this enhancement as Nile and Gillespie (2012) found that in the guinea pig bladder urothelium/lamina propria strips, muscarinic agonist-induced PGE_2 release that was attenuated by an M2 antagonist. As mentioned earlier, pre-treatment with 30% ethanol has removed the majority of urothelial M2 receptors, intermediate and basal cells which contain the COX 1 enzymes that are responsible for synthesizing prostanoids (Bschleipfer et al., 2007, de Jongh et al., 2009).

In addition, the action of ethanol on K^+ channels within the body is variable. In clinically relevant concentrations (10-100 mM) it can inhibit the BK channels in vascular tissues and in human IK channels expressed in Xenopus oocytes (Walters et al., 2000, Bukiya et al., 2011, Namba et al., 2005). In other tissues, such as the cultured guinea-pig detrusor smooth muscle cells, ethanol in concentrations of 0.3% enhances BK activity promoting detrusor relaxation (Malysz et al., 2014). There remains a possibility that low levels of ethanol in the tissues after pre-treatment with 30% ethanol have inhibited the BK channels in urothelium/lamina propria blood vessels affecting afterhyperpolarization contributing to enhanced muscarinic responses which additionally suggests that the blood vessels in the urothelial/lamina propria contribute to the contractile activity in a meaningful way.

The enhanced contractile response of the detrusor to EFS and muscarinic stimulation could also be induced by remaining ethanol present in the detrusor tissue. Ethanol's actions on the TRPV1 channels, which have also been identified in the detrusor, could also be promoting the release of neuropeptides contributing to the enhanced responses (Maggi et al., 1984, Persson et al., 1991, Pang et al., 1995, Ost et al., 2002, Trevisani et al., 2002). For this particular tissue, increased production of prostaglandins by ethanol cannot be ruled out as a factor enhancing this response either as prostaglandins have been found to enhance nerve-mediated contractions in the cat and rabbit detrusor (Larsson, 1980, Husted et al., 1980). Moreover, ethanol on other tissues has been found to stimulate prostaglandin synthesis (Collier et al., 1975).

The similar responses found in the intact tissues pre-treated with 30% ethanol compared to control treated tissues could be due to an enhancement in NO and urothelially derived inhibitory factor (UDIF) release.

Also, pre-treatment with 30% ethanol enhanced the inhibitory effect of the urothelium/lamina propria on the detrusor by approximately 25%.

Ethanol has been found to increase NO in vascular tissue, and to put this in context, the TRPV1 channels found on sensory nerves in the pigs are positive for SP, NOS and other neuropeptides (Greenberg et al., 1993, Fathian-Sabet et al., 2001, Ost et al., 2002, Pidsudko, 2014). While the underlying urothelial/lamina propria structures are more exposed following treatment with 30% ethanol, it is possible that activation of the TRPV1 channels on sub-urothelial nerves and interstitial cells has enhanced NO release (in addition to SP). It has been found in rat bladder strips that capsaicin whose cellular target is TRPV1 channels, evoked NO release while removal of the urothelium/lamina propria diminished this release by 72% (Birder et al., 2001). Furthermore, the proposed target enzyme for NO is guanylyl cyclase which has been found throughout the bl

adder including the detrusor and vascular smooth muscle (Wakabayashi et al., 1993). The guanylyl cyclase found in the detrusor and vascular smooth muscle could be the recipient of the proposed ethanol-induced NO, diffusing down from the urothelium and contributing to the non-significant response that was found in the intact tissue.

With regards to urothelially derived inhibitory factor (UDIF), it was found in intact pig bladder strips that pre-incubation with isoprenaline (β-adrenoceptor agonist that increases cAMP) was more potent at inhibiting carbachol mediated contractions compared to detrusor strips which was attributed to UDIF (Murakami et al., 2007). As mentioned earlier, ethanol exposure on tissues has been found to be a potent activator of adenylate cyclase activity and cAMP which may be increasing the amount of UDIF produced by the urothelium/lamina propria which may also be reducing the overall intact contractile response to carbachol (Hoffman and Tabakoff, 1990). Taken together, pre-treatment of the bladder with 30% ethanol alters the responses to non-receptor mediated contractile activity, parasympathetic cholinergic and purinergic stimulation.

The relaxation response of the detrusor to β-adrenoceptor stimulation with isoprenaline

The pig bladder relaxes in response to isoprenaline by stimulation of the bladder β-adrenoceptors (Murakami et al., 2007). For these experiments, stimulation of the adrenoceptors produced relaxation in the detrusor for all tissues examined. Following pre-treatment with 10%

or 30% ethanol, there were no changes to the relaxation response of the detrusor indicating that ethanol has not interfered with the sympathetic transmission.

Implications for IC/BPS

10% ethanol

Ethanol appears to have multiple effects on the bladder tissue. For overall bladder function, it appears that for tissues treated with 10% ethanol, this concentration has penetrated the GAG layer and has disrupted the outer umbrella cell layer. The lipophilic portion of the ethanol molecule may have interfered with many receptors usually found in these cells. Based on results and speculation, it does not appear likely that 10% ethanol has diffused any further than the basal cells. The initial enhancement of ATP that was found during treatment is most likely contributing to some of the mild pain experienced by non-ulcerating IC/BPS vehicle control subjects during intravesical instillation with 10% ethanol (Payne et al., 2005, Chen et al., 2005b). For some patients with ulcerative IC/BPS, the enhanced ATP release would be able to diffuse through the "leaky" urothelial layer with greater ease and interact with the elevated populations of P2X receptors greatly enhancing the purinergic response (Tempest et al., 2004, Leiby et al., 2007, Birder, 2014).

The speculative findings that 10% ethanol may have gone no further than the basal layer of urothelial cells is based on the reductions found in basal and stretch release of ATP and the increase in the amplitude of spontaneous contractions that was found in the urothelial/ lamina propria tissue only. The decreased levels of ATP in basal and stretch conditions may provide some welcome relief to IC/BPS patients who have increased urothelial ATP release in basal and stretch conditions (Kumar et al., 2007). The contractile responses to EFS, purinergic and muscarinic stimulation in the urothelium/lamina propria and detrusor and intact tissue have remained unaffected by pre-treatment with 10% ethanol. Taken together, these findings suggest that only minor irritation of the urothelium/lamina propria has occurred. However, the minor irritation observed in our representation of "full thickness bladder tissue" could be significantly enhanced in IC/BPS patients depending on the severity of their condition and the state of the urothelium along with the barrier components (Tomaszewski et al., 2001, Shie and Kuo, 2011).

It is important to note that as a vehicle, this concentration of ethanol may not be sufficient to enhance the penetration of drugs that target underlying structures, such as nerves in the bladder. This is additionally supported by Gordon et al. (2003) who could not find any detectable levels of ethanol in breathalyser data in IC/BPS patients after 30-minutes while 10% ethanol was present in the bladder. This suggests that 10% ethanol did not penetrate far enough to reach the sub-urothelial blood supply.

30% ethanol

For overall observations of bladder function following the intravesical application of 30% ethanol, this concentration has removed the GAG layer and much of the urothelial cell layer (umbrella, intermediate and basal cells) along with its receptors. This is consistent with histological evidence found in rat bladders using the same concentration (Byrne et al., 1998). It seems reasonable to assume based on the results of this study, that lower undetermined non-toxic concentrations of ethanol have further diffused through the lamina propria to the sensory nerves in the bladder wall creating a neurogenic inflammatory response by activating the TRPV1 channels on sensory nerves that have also been implicated in detecting heat (Bevan and Szolcsanyi, 1990, Holzer, 1991, Szallasi and Blumberg, 1999). This is evidenced by both vehicle control groups experiencing burning sensations (including pain) while undergoing intravesical treatment with 30% ethanol that took <2weeks to resolve (de Seze et al., 1998, Wiart et al., 1998). Although the subjects in the previously mentioned studies have neurogenic detrusor hyperreflexia, the consequence of this concentration in bladders affected by IC/BPS would be enhanced to a much greater degree. While ethanol is in contact with these bladders, the robust increase in ATP that is being dispersed could potentially be creating severe bladder pain and enhanced contractile activity as it interacts with the upregulated P2X1, P2X2 and P2X3 receptors (Tempest et al., 2004, Neuhaus et al., 2012).

Intense bladder sensation as a result of ethanol treatment is in agreement with Fagerli et al. (1999) who by personal communication with Chancellor during a pilot study for patients with IC/BPS, stated that ethanol at this concentration was not tolerated. The consequence of reduced ACh levels observed during treatment and in basal conditions after treatment is unknown. However, this may affect the regulation of other urothelial transmitters and alter sensory aspects in the bladder. (Braverman et al., 2007, Kullmann et al., 2008b, Daly et al., 2010, Moro et al., 2011).Moreover, anti-muscarinic therapy in the bladder is reportedly more effective in

the storage phase of the micturition cycle when parasympathetic activity is absent (Yoshida et al., 2004).

As stretch-induced ACh release remained unaffected by treatment, the cholinergic signal produced by urothelium/lamina propria would almost certainly be already heightened as Mukerji et al. (2006b) found elevations in the expression of M2 receptors on the sub-urothelial interstitial cells in IC/BPS. In addition, there would be a higher degree of neurogenic inflammation in IC/BPS patients as a result of ethanol acting on TRPV1 channels on the sensory afferents as Pang et al. (1995) identified increases to SP containing fibres in IC/BPS bladders in the sub-urothelial space. This is not including the easy passage for noxious stimuli including K+ after the removal of the protective GAG layer and urothelial cells as well as the reported increase in prostaglandin activity found in ulcerative IC/BPS patients (Parsons et al., 1998, Narumiya et al., 1999, Wada et al., 2015).

The contractile enhancements found in the detrusor in response to muscarinic stimulation may or may not be enhanced further in IC/BPS patients as Pang et al. (1995) additionally found that SP containing fibres were not significantly changed in the detrusor of IC/BPS patients compared to control subjects. Moreover, it is uncertain whether increases in NO or UDIF mediate the unaltered carbachol (muscarinic agonist) based contractions found in the intact samples for this study and the impact in IC/BPS would be unknown due to the already elevated levels of eNOS and iNOS (Ying and Hofseth, 2007, Logadottir et al., 2013, Jhang et al., 2016). By and large, for bladders affected by IC/BPS, 30% ethanol would most likely be producing considerably more pain during treatment. After treatment, pain and neurogenic inflammation originating from the urothelium/lamina propria and increases to purinergic activity due to elevated P2X1 receptors in the detrusor would most likely be intensified compared to the un-diseased bladder making it largely intolerable for these patients. However, it appears that this concentration would allow for drug diffusion to the sensory fibres in the bladder wall.

Summary

In summary, 10% ethanol as a vehicle produced mild damage to the urothelium by the enhancement of ATP release and LDH activity from the urothelial surface during treatment. The isolated urothelium/lamina propria following pre-treatment with 10% ethanol had diminished releases of ATP under basal and stretch conditions, undetected ACh release under basal conditions while the amplitude of spontaneous contractile activity was enhanced. There

were no further changes to the isolated tissues or bladder function as a result of pre-treatment with 10% ethanol. In contrast, 30% ethanol produced significant damage to the urothelial layers that was evident by the robust release of ATP, enhanced LDH activity and diminished ACh release during treatment. Histological analysis revealed that 30% ethanol was specific for the urothelial cell layers as their width was reduced by 75%. For the isolated urothelium/lamina propria following pre-treatment, there were decreases to ATP under basal and stretch conditions while ACh release was undetected under basal conditions which was similar to the effects of 10% ethanol. The urothelium/lamina propria also had a depressed response to purinergic stimulation while KCl and muscarinic stimulation enhanced the contractile response. The isolated detrusor had enhanced contractile responses to muscarinic stimulation and EFS.

In conclusion, the use of 10% ethanol as a vehicle produces mild side effects mostly by the release of ATP. However, it is uncertain whether this concentration is effective for RTX to reach the sub-urothelial layers. On the other hand, 30% ethanol, by the sloughing of the urothelium, produces considerable side effects which are consistent with the literature and can persist for up to two weeks. Although there are initial side-effects attributed to 30% ethanol, there is no doubt that this concentration would allow drug diffusion to the sub-urothelial layer. The observed effects for both concentrations of ethanol would be largely intensified in IC/BPS.

Chapter 5:
The effects of resiniferatoxin and capsaicin on the function of isolated pig bladder

5.1 Introduction

Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) is a naturally occurring vanilloid containing the functional vanillyl group in its formula. Vanilloids belongs to a class of organic chemicals referred to as proto-alkaloids. Capsaicin is derived from the capsicum family and is the main element responsible for the hot, pungent taste of chili when ingested or when applied topically to the skin. The molecular structure of capsaicin is composed of a vanillyl group, an amide bond and a long hydrophobic side chain **(Figure 5.1)**, (Hayman and Kam, 2008, Sharma et al., 2013, Elokely et al., 2016)**.** Resiniferatoxin (resiniferanol 9,13,14-orthophenylacetate) (RTX) is an extract from the Moroccan cactus-like plant *Euphobia resinifera* and is an ultrapotent capsaicin analogue. Resiniferatoxin is structurally similar to tumor-promoting phorbol esters that act via protein kinase C (PKC) yet, does not to promote the formation of tumours nor compete efficiently for the phorbol ester binding site on PKC. The key difference between phorbol esters and RTX is that phorbol esters have a free OH group at C20, essential for phorbol like activity in contrast to RTX which is instead esterified with a vanillyl group at the same position and is therefore termed a vanilloid (Hergenhahn et al., 1975, Winter et al., 1990, Appendino and Szallasi, 1997). Like capsaicin, RTX can be subdivided into three regions consisting of a vanillyl group, an ester bond and a polyring group **(Figure 5.1)**, (Elokely et al., 2016).

Capsaicin and RTX are not soluble in water, hence only alcohols, and organic solvents are used to solubilize them. (Hayman and Kam, 2008, Bode and Dong, 2011, Frias and Merighi, 2016). For intravesical use in the bladder, RTX is generally dissolved in 10% ethanol, and capsaicin is usually dissolved in 30% ethanol (Chancellor and de Groat, 1999). As a point of reference, on the Scoville scale which is a measure of pungency, pure capsaicin measures sixteen million Scoville heat units (SHU). RTX measures sixteen billion SHU (1000 times higher than capsaicin), and ingestion of less than 10 g can be fatal (Elokely et al., 2016, Chilli World.com, 2018).

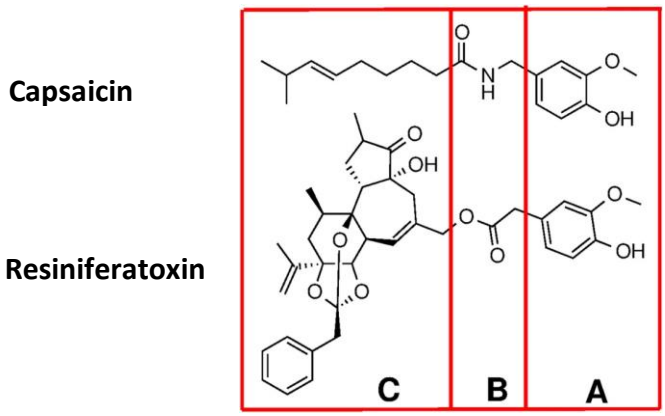

Figure 5.1: Structural subdivisions of capsaicin and resiniferatoxin (reproduced with permission from the publisher, (Elokely et al., 2016)).

Target tissue

In general, RTX and capsaicin bind to a select group of sensory nerve fibres and transmit noxious afferent information (pain or itching) via the capsaicin receptor also known as the TRPV1 channel to the central nervous system (Szallasi, 1996, Caterina et al., 1997). The transient receptor potential vanilloid (TRPV)1 channel reportedly consists of 838 amino acids that has six transmembrane domains with an additional short hydrophobic region between transmembrane domain five and six **(Figure 5.2)**, (Caterina et al., 1997).

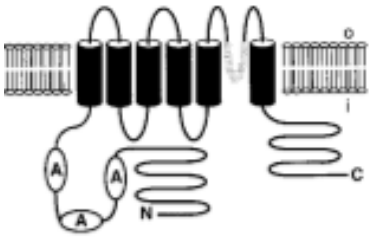

Figure 5.2: The predicted structure of the TRPV1 channel (reproduced with permission from the publisher, (Caterina et al., 1997)).

Capsaicin-sensitive TRPV1 channels are found on sensory Aδ and C-fibres, specifically, nociceptors such as poly-modal nociceptors, silent nociceptors and some warm receptors. Mechanical Aδ and C nociceptors and various other types of low threshold mechanoreceptors including cold receptors, cholinergic and adrenergic neurons are insensitive to capsaicin. (Gamse et al., 1982, Holzer, 1991, Szolcsanyi et al., 1998). Resiniferatoxin also stimulates TRPV1 channels and therefore acts on the same subset of neurons that capsaicin does, but it is significantly more potent (Winter et al., 1990, Szallasi and Blumberg, 1989).

Pharmacology

Capsaicin binding to the TRPV1 channel opens the pore leading to predominantly a calcium (Ca^{2+}) influx (Jung et al., 1999). The estimated permeability sequence produced by capsaicin acting on the TRPV1 channel is Ca^{2+}> magnesium (Mg^{2+})> guanidinium> potassium (K^{+})> sodium (Na^{+})> choline (Szallasi, 1996). In the rat, it was found that capsaicin stimulation of freshly isolated afferent neurons from dorsal root ganglia (DRG) resulted in Ca^{2+} influx which in-turn liberated intracellular Ca^{2+} via ryanodine receptors but not inositol triphosphate (IP3) receptors. (Huang et al., 2008).

Given that capsaicin and RTX are lipid soluble, it has been suggested that extracellular application activates the TRPV1 channel by diffusion through the cell membrane and binding to at least 2 sites in the cytosol (Jung et al., 1999). Furthermore, TRPV1 channel immunostaining techniques found functional TRPV1 channels co-localizing with the endoplasmic reticulum (ER) in rat DRG neurons. The release of Ca^{2+} from intracellular stores seems to be concentration-dependent as lower concentrations of capsaicin permit influx from the extracellular space whereas much higher concentrations are required for intracellular release presumably by interaction with the TRPV1 channels found on the ER (Gallego-Sandin et al., 2009). In rat DRG neurons, guanosine cyclic 3',5'-phosphate (cGMP) is also elevated in response to capsaicin which was dependent on extracellular Ca^{2+} (Wood et al., 1989).

In a similar fashion to capsaicin, RTX depolarizes sensory nerves, elevates cGMP levels and allows the influx of cations (predominantly Ca^{2+}), and release of Ca^{2+} from intracellular stores (Winter et al., 1990, Olah et al., 2001) As RTX is much bulkier and more lipophilic than capsaicin, tissue penetration is much slower (Appendino and Szallasi, 1997). Maggi et al. (1990b) found that the time course of action of RTX was much slower when compared to capsaicin. Additional electrophysiology studies confirmed that the RTX-induced current had a much slower onset that was persistent compared to the rapid transient ion current evoked by capsaicin (Winter et al., 1990).

Experiments examining binding and Ca^{2+} uptake in cultured rat DRG sensory neurons identified that RTX was 10,000 fold more potent at binding to the TRPV1 channel rather than Ca^{2+} uptake, this is in contrast to capsaicin which is much more potent at inducing Ca^{2+} uptake when compared to its binding affinity (Acs et al., 1996). Although RTX has been portrayed as

being more potent than capsaicin, binding affinities are not the same for all species. Pharmacology studies that describe RTX binding with human TRPV1 channels identified a twenty to forty-fold lower binding affinity when compared to the rat TRPV1 channels (Acs et al., 1994). Of interest and in regards to the seemingly lower affinity RTX has for human TRPV1 channels, rimcazole, a neuroleptic drug that alleviates neuropathic pain syndromes, actually facilitates RTX binding to human vanilloid receptors where as in the rat it inhibits binding (Acs et al., 1995).

Mechanism of action

Capsaicin's actions are unique as it produces a two-fold effect of initial stimulation followed by a perpetual refractory state traditionally termed "desensitization". (Szallasi and Blumberg, 1999) It is the lasting "desensitization" that has clear therapeutic potential for reducing neuropathic pain and treating other pathological conditions in which the capsaicin-sensitive fibres are thought to play a role. (Jancso et al., 1980, Frias and Merighi, 2016)

Firstly, following TRPV1 activation by capsaicin, the cytosolic increase in Ca^{2+} leads to depolarization of the afferent neuron and discharge of action potentials that is perceived as burning pain (Bevan and Szolcsanyi, 1990, Holzer, 1991, Szallasi and Blumberg, 1999). Secondly, the peripheral terminals, in turn, release a variety of neuropeptides such as substance P (SP), somatostatin and calcitonin gene-related peptide (CGRP) initiating the cascade of neurogenic inflammation and the initial stimulatory events caused by capsaicin (Szallasi and Blumberg, 1989, Szallasi, 1996).

The extent to which peptides are liberated by capsaicin is dependent on the dose, time of exposure and interval between doses. At high doses or prolonged exposure to capsaicin, desensitization ensues and following applications become less pronounced or fail to produce any effects (Holzer, 1991). High doses of capsaicin are also associated with non-specific effects such as a loss of responsiveness to additional chemical and physical stimuli. (Kenins, 1982, Szolcsanyi, 1987, Maggi et al., 1989b, Amann, 1990).

The mechanism by which vanilloids liberate neurotransmitters is unclear. However, the release of these peptides appears to be by a direct mechanism dependent on extracellular Ca^{2+} rather that an axonal reflex (Gamse et al., 1979, Maggi and Meli, 1988, Szallasi, 1996).

The exhaustion of neuropeptides was thought to be primarily responsible for the unresponsiveness of the sensory nerves and resulting pain relief achieved with capsaicin and in fact may be responsible for the acute analgesic effect. However, studies have determined desensitization to be independent of peptide depletion, and the persistent longer effects seen by treatment with capsaicin are complex and are likely due to several factors that include receptor desensitization, de-functionalization of the afferent fibres and neurotoxicity. The degree of desensitization appears to be concentration-dependent and can last for several days to months (Gamse et al., 1979, Lembeck and Donnerer, 1981, Gamse et al., 1982, Maggi et al., 1990a, Polydefkis et al., 2004, Anand and Bley, 2011).

De-functionalization may begin by excessive levels of Ca^{2+} and other cations accumulating in the afferents. Han et al. (2007) observed a drastic increase in intracellular Ca^{2+} levels after treating HEK rat TRPV1/C2 cell lines with capsaicin. The elevated levels of Ca^{2+} were found to be detrimental to cellular processes as cellular protein synthesis was inhibited along with disassembly of microtubules. Microtubule disassembly is harmful to the cell as it causes vesicle accumulation and impairs neurotransmitter release. Furthermore, Gamse et al. (1982) noted long-term biochemical and functional changes in rat sciatic sensory neurons after the application of capsaicin. Those changes consisted of a loss of axonal transport and impaired impulse conduction.

The neurotoxic effects of capsaicin are well documented and are thought to occur by the accumulation of Ca^{2+} and Na^{+} followed by an influx of water producing osmotic swelling, damage and eventual lysis (Marsh et al., 1987, Wood et al., 1988, Szallasi, 1996). In humans the topical application of 1.8 g of 0.1% capsaicin over a period of 24-hours produced reversible denervation of the sensory fibres in the epidermis and dermis that regenerated over a period of several months. (Polydefkis et al., 2004)

The significant key difference that RTX has in contrast to capsaicin is that it generally seems to favour desensitization without prior excitation. For example, it was found when >0.1 µM RTX was added to isolated rat bladder strips it produced contraction (initial stimulatory event) similar to capsaicin. However, much lower concentrations that did not elicit a contractile response inhibited further contractions to subsequent challenges of 1 µM of capsaicin (Maggi et al., 1990b). A single subcutaneous injection of RTX (0.5mg/kg) in rats also induced transient insensitivity to mechanical stimuli (Xu et al., 1997). An interesting theory put forward by

Avelino et al. (1999) after observing the potent desensitizing yet less irritating action of intravesical RTX (100nM) on rat bladder was that RTX might only act on low threshold fibres encoding micturition at a physiological range, whereas capsaicin, acts on both low and high threshold fibres. The high threshold fibres respond to signals of noxious pain.

Resiniferatoxin can down-regulate the expression of its receptors, and subcutaneous administration of RTX in the rat produces an 80-90% loss of binding sites in the DRG and trigeminal ganglia that was evident 24-hours after treatment (Szallasi and Blumberg, 1992). A loss of RTX binding sites has also been found in the rat bladder 24-hours after subcutaneous administration which was long-lasting (>2 months) in contrast to non-recovery of binding sites in spinal cord membranes, most likely reflecting desensitization and neurotoxicity in these tissues (Goso et al., 1993).

Although the immediate response of RTX produces neurotransmitter outflow from afferent neurons, the neural damage is apparent after prolonged or repeated brief exposure (Maggi et al., 1990b, Winter et al., 1990). On a cellular level, cultured cells exposed to RTX experienced cell bursting that was due to the effects of accumulated Ca^{2+} after 1-2 hours. The time frame for cellular disruption was found to be concentration-dependent (Olah et al., 2001).

The effect of capsaicin on the bladder

There are species-related differences with regards to capsaicin's activity in the bladder (Maggi et al., 1987b). However, the majority of our present knowledge has been obtained in rat studies. In adult rat bladders, the initial administration of capsaicin produces concentration-dependent contractions followed by a lasting refractory state that is insensitive to repeated applications of capsaicin (up to 3-hours from the 1st application) (Santicioli et al., 1986, Maggi et al., 1990a, Maggi et al., 1990b). The contraction induced by capsaicin occurs mostly due to the release of neuropeptides, notably SP, while detrusor NK1 and NK2 receptors were found to play a role (Maggi et al., 1987a, Amann, 1990, Saitoh et al., 2007). In addition to releasing numerous peptides, capsaicin treatment of rat bladder strips induces nitric oxide (NO) release that is due partly to the activation of capsaicin-sensitive nerves and directly from the urothelium via urothelial TRPV1 channel activation. (Birder et al., 1998)

Maggi et al. (1987c) identified that after rat bladders were exposed to capsaicin for 5-minutes, a consistent reduction of SP occurred over a period of 6-hours. After exposure to high doses of

capsaicin, contraction induced by dimethylphenylpiperazinium (DMPP), which activates the ganglionic nicotinic receptors, was significantly lower demonstrating the non-specific effects of capsaicin (Maggi et al., 1989b).

Urodynamically, the application of capsaicin to the rat bladder increases in volume threshold 30-minutes after treatment with no significant effect on the amplitude of micturition contractions (Maggi et al., 1990b). Increases in the micturition threshold in rat bladders treated subcutaneously with capsaicin have been observed for up to thirty days (Santicioli et al., 1985). These findings suggest that capsaicin-sensitive afferents are involved in regulation of the micturition threshold. A Histological analysis on the rat bladder found that 1mM of capsaicin dissolved in 30% ethanol produced acute mucosal injury, submucosal oedema, vascular ectasia and congestion as well as thinning and denuding of the urothelium with significant disruption of the glycosaminoglycan (GAG) layer. Similar findings were reported with 30% ethanol alone as reported in the previous chapter, but not to the same degree as both ethanol and capsaicin. Recovery occurred over a period of one week (Byrne et al., 1998).

In humans, the initial intravesical administration of 1 and 10 μM capsaicin in <0.1% ethanol initially resulted in a warm to burning sensation in the supra-pubic region with decreased pressure thresholds for micturition that were concentration dependent. The warm burning sensation persisted for 10-120 minutes post micturition. Two to three days post-capsaicin instillation, patients who had hypersensitive disorders reported the complete disappearance or marked attenuation of their symptoms (pain, urgency and frequency) which lasted for four to sixteen days. No additional side effects were reported. Interestingly, a patient who had urinary symptoms such as nocturia, frequency and dysuria due to benign prostatic hyperplasia did not report any symptom improvement. This suggests that hypersensitive disorders of the bladder have a neuroinflammatory component mediated by capsaicin-sensitive afferent fibres. (Maggi et al., 1989a)

The use of capsaicin for IC/BPS

Intravesical application of capsaicin has mainly been targeted at patients with neurological disorders of the bladder who experience urinary symptoms thought to be driven by C-fibre input, usually ineffective for initiating micturition in normal circumstances. (de Groat et al., 1990, Chancellor and de Groat, 1999) However, capsaicin has additionally been found to provide clinical benefit for patients with interstitial cystitis/Bladder pain syndrome (IC/BPS)

even though its pathogenesis is not generally thought to be of neurological origin, but a reported C-fibre involvement (Pang et al., 1995, Cruz et al., 1997, Hanno et al., 2012). Maggi et al. (1989a) provided initial evidence of the clinical benefit of consecutive intravesical instillations of capsaicin for bladder hypersensitivity disorders using low concentrations of capsaicin. Following on from Maggi et al. (1989a), Barbanti et al. (1993) also described clinical benefit of the intravesical use of capsaicin for patients with hypersensitive bladders with improvements reported in pain frequency and nocturia. The benefits persisting for up to fifteen days.

In a randomized placebo-controlled study by Lazzeri et al. (1996), patients with bladder pain and non-ulcerating IC/BPS were put into a treatment group and a placebo group. The treatment group received intravesical instillation of 10uM of capsaicin (solvent not described) twice a week for one month while the placebo group received saline solution warmed to 42° C which mimics the warm burning sensation produced by capsaicin. All patients in the treatment group experienced a burning sensation on instillation that progressively became less intense with successive instillations. Significant improvements were noted in pain, frequency and nocturia with 70% of the treated participants reporting an improvement in the quality of life immediately after the study. Approximately 50% of these participants required no medications six months after treatment. Although patients experienced discomfort from capsaicin instillation, Lazzeri et al. (1996) has suggested that a larger dose may further improve symptoms associated with IC/BPS.

For higher concentrations of capsaicin (1-2 mM) delivered intravesically to the bladder to treat other disorders, 30% ethanol is commonly used as the solvent. However, it has been reported that this concentration of ethanol has not been well tolerated in IC/BPS patients (Chancellor and de Groat, 1999, Fagerli et al., 1999). Concerned with this solvent issue for this subset of patients, higher doses of capsaicin of up to 250 μM dissolved in 1% ethanol have been used with no associated complications by Fagerli et al. (1999) in five clinically diagnosed patients with IC/BPS. The majority of patients reported improvements in pain and symptoms with a trend of decreased urinary SP post-treatment.

Higher doses of capsaicin (1 mM) dissolved in 30% ethanol have been used in patients with hypersensitivity disorders of the bladder. A single high dose of 1 mM of capsaicin dissolved in 30% ethanol delivered intravesically produced a transient worsening of symptoms for approximately two weeks after treatment. This was followed by marked improvements in

urinary frequency, urge incontinence, maximal cystometric capacity and first desire to void. These improvements were maintained for at least ≥3 months after treatment. The clinical benefit of this study is thought to be due to a block of C-fibre input (Cruz et al., 1997). Similar results were achieved in another study that delivered the same concentration of capsaicin with 30% ethanol intravesically into hypersensitive bladders with significant improvements reported in bladder capacity (Soontrapa et al., 2003).

The effect of RTX on the bladder

The initial administration of RTX to rat bladders evokes a concentration-dependent and prompt outflow of neurotransmitters such as SP and CRGP. As mentioned earlier, RTX in similar concentrations to capsaicin triggers contractile activity. At lower concentrations (500-1000-fold less) that did not elicit a contraction, reduction of contractile activity was evident to subsequent challenges of capsaicin that demonstrates the potent predominantly desensitizing properties of RTX. Treatment with RTX (10nM) is urodynamically much the same as capsaicin (10uM) as it induces a significant increase in volume threshold for reflex micturition with no effect on the amplitude of micturition albeit at a lower concentration (Maggi et al., 1990b). Avelino et al. (1999) instilled 100 nM RTX in 10% ethanol in rat bladders for 30-minutes and found *c-fos* (Fos-protein evaluates nociceptive input) immunoreaction in spinal segments (L5-S1) was produced two-fold more than simple urethral catheterization in lamina I and the intermediolateral grey matter with no effect in the dorsal commissure 2-hours after treatment. This contrasts with 1 mM of capsaicin which increased the Fos-immunoreactive cell count by ten-fold in the lamina I, five-fold in the intermediolateral grey matter and by four-fold in the dorsal commissure in a previous experiment (Avelino et al., 1998). Furthermore, it was ascertained that 100 nM was the saturating concentration that could not be enhanced by increasing the dose to 1000 nM (Avelino et al., 1999).

Studies of normal human bladder noted an absence of warm burning sensations at the suprapubic or urethral level during instillation of 10nM of RTX. Even at 100nM RTX produced no systemic or local side effects when applied intravesically to spinal cord injured patients (Lazzeri et al., 1997, Giannantoni et al., 2002).

The use of RTX for IC/BPS

Results from patients with IC/BPS, bladder pain or hypersensitivity treated intravesically with RTX have been conflicting. In a randomized placebo-controlled study, patients with bladder hypersensitivity and bladder pain without ulceration received a single intravesical dose of 10nM of RTX in 0.1% ethanol while the placebo group received a saline solution. A light warm burning sensation was experienced in the treatment group but not in the placebo group. In the treated group thirty days after treatment, there was a significant improvement in pain, nocturia and frequency and 66% of the patients reported an improvement in the quality of life. At three months post-RTX treatment, only 33% of the same treated patients did not require medication to control their symptoms (Lazzeri et al., 2000). Apostolidis et al. (2006) treated patients with bladder symptoms including bladder pain with 50nM RTX dissolved in 10% ethanol. Urodynamic parameters and quality of life were significantly improved for up to six months. Some of these patients experienced pain on instillation despite pre-treatment with lidocaine.

On the other hand, not so attractive results regarding the use of RTX in clinically diagnosed non-ulcerative IC/BPS patients has been observed by Chen et al. (2005b). In the randomized, double-blind placebo-controlled trial, patients received intravesical instillation of either 50 nM, 100 nM RTX dissolved in 10% ethanol while the placebo group received 10% ethanol. The majority of all patients in patient groups experienced pain upon instillation although no patient discontinued the treatment nor were any adverse events reported. These findings lead the investigators to conclude that RTX at the above mentions concentrations was deemed safe in this subset of patients. Disappointingly, the follow-up results at week four and week twelve on symptomology provided no significant improvements.

Similar findings were reported by Payne et al. (2005) in another large randomized, double blind placebo-controlled trial consisting of clinically defined non-ulcerating IC/BPS patients. There were four groups in this study that consisted of 10 nM , 50 nM and 100 nM RTX dissolved in 10% ethanol and a placebo group that received an instillation of 10% ethanol. There was a dose-dependent increase in pain and urgency in all groups including placebo group on instillation of RTX despite pre-treatment with lidocaine. Another unusual finding was a transient dose-dependent increase in systolic blood pressure related to pain at instillation, while over the twelve-week study period post-instillation, no significant improvements were noted in pain, frequency, urgency or nocturia.

Lazzeri et al. (2004a) explored prolonged infusion of 10 nM RTX by a temporary in situ drug delivery system installed in the bladders of five clinically diagnosed IC/BPS patients. The bladder was continually infused for ten days in which all patients reported worsening of symptoms that improved after three days. During the study period post-instillation, all patients experienced long-lasting clinical benefit for up to three months with improvement in pain, frequency and nocturia.

5.2 Objectives

The purpose of this study was to investigate the effect of luminal RTX and capsaicin treatment on the integrity and function of the pig bladder. Specific aims were;

1. To quantify urothelial adenosine 5'-triphosphate (ATP) and acetylcholine (ACh) release during treatment with RTX and capsaicin.
2. To examine the structure of the urothelium/lamina propria after pre-treatment with RTX and capsaicin.
3. To quantify mediators released by the urothelium/lamina propria in basal and stretched conditions after pre-treatment.
4. To investigate the effect that RTX and capsaicin has on efferent nerve-mediated responses of the detrusor after pre-treatment.
5. To explore changes in contraction and relaxation responses of the urothelium/lamina propria, detrusor or whole bladder strips as a result of pre-treatment with RTX and capsaicin.

5.3 Materials and methods

Animals

Fresh porcine bladder tissue from mature sows (>1-year-old) was retrieved from the local abattoir (Highchester Meats, Beaudesert, Queensland) and were immediately placed in cold Krebs bicarbonate solution (NaCl 118.4mM, NaHCO3 24.9mM, KCl 4.7mM, CaCl2 1.9mM, MgSO4 1.2mM, glucose 11.7mM) for transport back to lab facilities.

Tissue Preparation

After the urethra, ureters and excess tissues were removed from the bladder, the bladder was opened by dissection from base to dome and was laid flat on a dissection board. Whole bladder sections from the dome were isolated and were mounted in modified Ussing chambers containing gassed (5%CO_2/95%O_2) Krebs-bicarbonate solution and kept at 37°C as described by Smith et al. (2014). 50 nM RTX dissolved in 10% ethanol or 1 mM capsaicin dissolved in 30% ethanol was prepared and placed on the luminal side of the bladder tissue to incubate for 30-minutes reflecting clinical intravesical treatment. For each RTX and capsaicin treatment, a matched vehicle control incubation was also conducted (i.e. 10% or 30% ethanol), **(see chapter three, Figure: 3.1)**.

Sampling of effluent

After treating the tissue for 30-minutes, a sample of the luminal medium was collected and was stored in a -30°C freezer for later analysis of urothelial ATP, ACh and lactate dehydrogenase (LDH) activity using commercially available kits (described in chapter two). The remaining treatment effluent was discarded.

Organ bath preparation

The tissue was then retrieved from the Ussing chambers and was washed with warm **(**37°C) Krebs-bicarbonate solution. Control and treated tissues were sectioned into three strips each including a urothelium/lamina propria strip that was dissected from an intact strip, a denuded detrusor strip, and an intact strip. Each strip was anchored in an individual organ bath containing gassed (5%CO_2/95%O_2) Krebs-bicarbonate solution kept at 37°C. In the organ baths, the tissues were connected to an isometric force transducer and tension was recorded by

a Powerlab 8/30 recording system (ADInstruments Ltd.) which was analysed by Lab Chart (version 7.0.3) software (ADInstruments Ltd.). In preparation for the following experiments, the tissues were washed every 15-minutes and were allowed to equilibrate for 1-hour under a resting tension of 150 mN **(see chapter four, Figure:4.2)**.

Spontaneous activity

After the equilibration period, the spontaneous activity of the urothelium/lamina propria, and detrusor was investigated. Amplitude was recorded from peak to trough of a contraction and frequency was recorded as contractions that exceeded 30% of the peak amplitude per minute. Adapted from (Imai et al., 2001).

Functional studies using pharmacological agents

To begin the functional studies, both RTX or capsaicin pre-treated tissues along with their matched vehicle control tissues were exposed to ATP (1 mM) and KCl (60 mM) to examine the purinergic component and non-receptor mediated contractile muscle activity respectively. After peak contraction was observed following ATP, the tissues were washed 2-3 times and were allowed to rest and re-equilibrate for 15-minutes before KCl was added to the organ bath. After a contractile plateau had been achieved in response to KCl, the tissues were washed another 2-3 times and allowed to re-equilibrate for another 15-minutes. Following on from ATP and KCl, a cumulative concentration-curve was created in response to the muscarinic agonist carbachol (10 nM – 100 μM). After the maximal dose and peak response to carbachol had occurred, detrusor tissues were washed every 15-minutes and allowed to re-equilibrate for another hour before beginning a cumulative concentration-curve in response to the β-adrenoceptor agonist isoprenaline (70 pM – 70 μM). Before recording the relaxation in response to isoprenaline, the tissues were initially pre-contracted with 30 μM carbachol (submaximal dose) and allowed to plateau for approximately 20-minutes.

The recorded responses of each tissue were taken by measuring the change in tension from baseline recordings before the addition of agonists. The changes in tension produced by the treated bladder strips were compared to their matched control strips.

Electrical Field stimulation (EFS)

For nerve stimulation studies, strips of RTX or capsaicin pre-treated detrusor strips with their matched vehicle control strips were isolated and individually anchored in organ baths as described previously. Following equilibration, the strips were electrically stimulated (20V, 1 ms pulse-width, 5 s train delivered every 100 s) to assess nerve-mediated contractile responses on the detrusor. The stimulatory frequencies consisted of 1 Hz, 5 Hz, 10 Hz and 20 Hz and the subsequent contractile responses were recorded and assessed. These same frequencies were repeated in the presence of 1μM atropine (muscarinic antagonist) and 10 μM α,β mATP (de-sensitizes P2X receptors) to investigate the muscarinic and purinergic contributions.

Urothelial mediator release

In a separate experiment, to assess urothelial mediator release under basal resting conditions and stretch conditions, three strips each of RTX or capsaicin pre-treated isolated urothelium/lamina propria tissues were tied together and individually anchored in organ baths along with their matched vehicle control tissues as described above. Following the equilibration period, 3 mL of warm Krebs-bicarbonate solution was added to the bath for a 2-minute period. After the 2-minute period, samples of Krebs surrounding the tissue were taken to represent bladder basal resting conditions. Immediately after collection, the bath was drained, and another 3 mL of warm Krebs-bicarbonate solution was added to the bath. Over another 2-minute period, the tissues were stretched from 150 mN to 1.5 N simulating the stretch that occurs during bladder filling. After the stretch was complete, samples were taken from solutions around the tissue to assess mediator release in stretched conditions. All samples were stored in a -30°C freezer until required for further analysis of ATP and ACh release using commercially available kits (described in chapter two).

Histology

Following the luminal treatment described above, intact bladder strips measuring approximately 1-2 mm wide were isolated and placed into neutral buffered formalin (10%) at 4°C for 24-hours. Tissues were then prepared into wax blocks and stained with hematoxylin and eosin. Light microscopy was used to assess any alteration to urothelial integrity that may have occurred as a result of treatment. The protocol and materials are described in chapter two.

Statistical Analysis

For these studies, data was expressed as mean ± standard error of the mean (SEM) and analysed by two-tailed T-Tests using Graphpad Instat (San Deigo, CA) version 3.10. Curve analysis was performed with multiple comparisons F-test using GraphPad Prism (San Deigo, CA) version (7.03). Significance was defined as *P<0.05, **P<0.01, ***P<0.001.

5.4 Results

Effect of luminal RTX and capsaicin on the release of chemical mediators and the integrity of the urothelium/lamina propria

The immediate effect that luminally applied RTX or capsaicin had on the urothelium was determined by quantifying ATP, ACh and LDH activity in the treatment effluent that was in direct contact with the urothelium. To rule out any interference of the treatment medium with the assay reagents, additional standard assay curves were created using the appropriate treatment medium.

While tissues were being treated with RTX, there were no significant alterations to ATP, ACh release or LDH activity when it was compared to vehicle control tissues **(Figure 5.3A,C &E)**. During treatment with capsaicin, there were no significant alterations to ATP release **(Figure 5.3B)**. However, there was a significantly greater enhancement in ACh release (capsaicin 12.4±0.9 µM vs. 30% EtOH 0.7±0.4 µM, $P<0.001$, $n=8$), **(Figure 5.3D)** while LDH activity **(Figure 5.3F)** remained similar to the vehicle controls.

Histological analysis using hematoxylin and eosin staining of the intact tissue assessed if any urothelial damage occurred following pre-treatment with RTX or capsaicin. Pre-treatment with RTX revealed some swelling of urothelial structures compared to vehicle control pre-treated tissues although urothelial thickness was unchanged to the vehicle. **(Figure 5.4E&F)**, **(Figure 5.6A)**. Tissues that had been pre-treated with capsaicin appeared similar to the vehicle with clear and visible sloughing of the urothelial cell layer that extended to the basal cell layer **(Figure 5.5E&F)**. Measurement of the remaining available urothelial layer revealed that pre-treatment with capsaicin did not significantly affect urothelial thickness when it was compared to the vehicle controls **(Figure 5.6B)**.

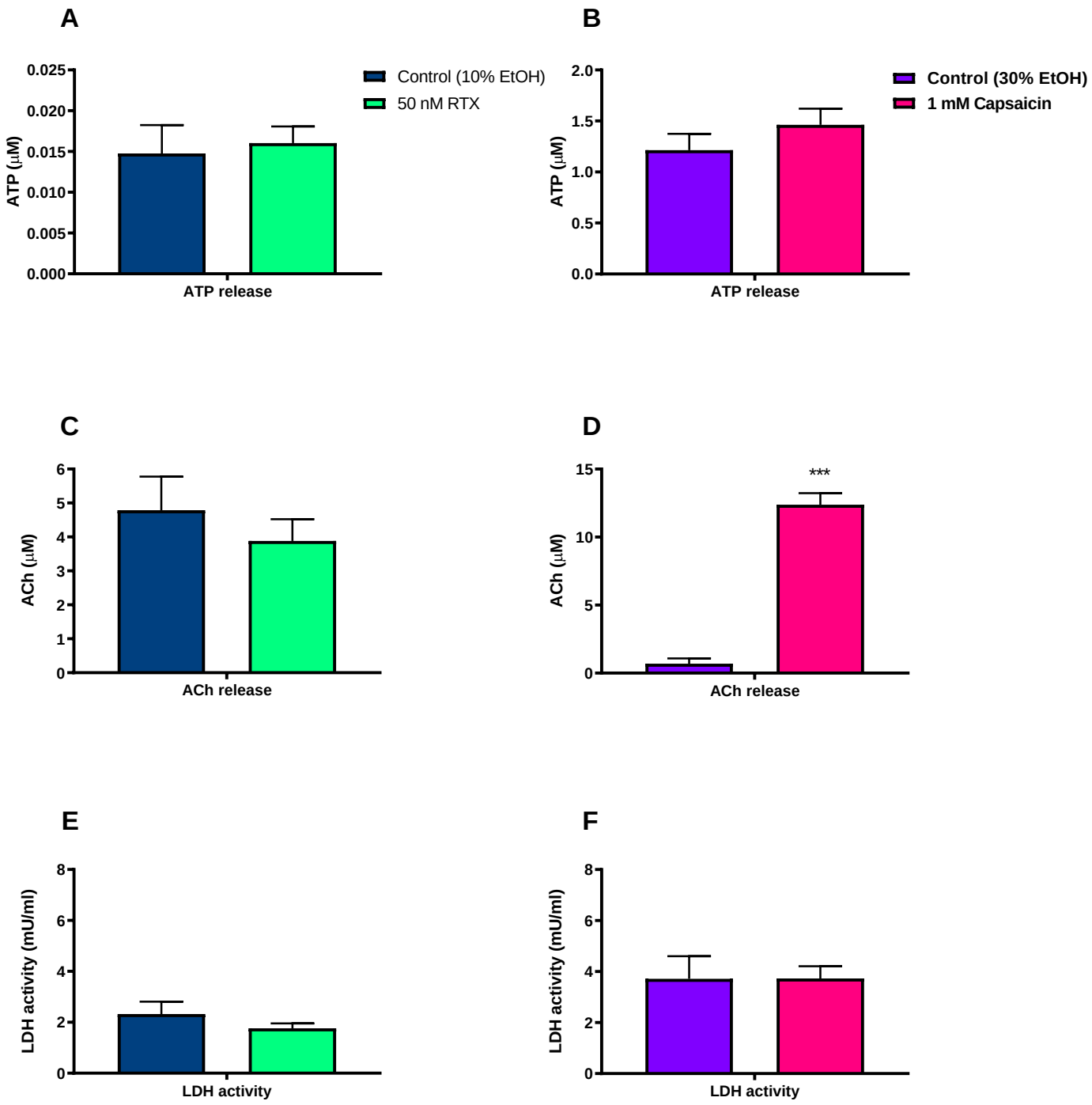

Figure 5.3: ATP release (A&B), ACh release (C&D) and LDH activity (E&F) in samples of treatment effluent collected following luminal pre-treatment of pig bladder tissues with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A,C&E) or the vehicle control for capsaicin (30% EtOH) and 1 mM Capsaicin (B,D&F). Data is represented as mean ± SEM (n≥5) analysed by an unpaired two-tailed t-test (*P<0.001, 30% EtOH vs. 1 mM capsaicin).**

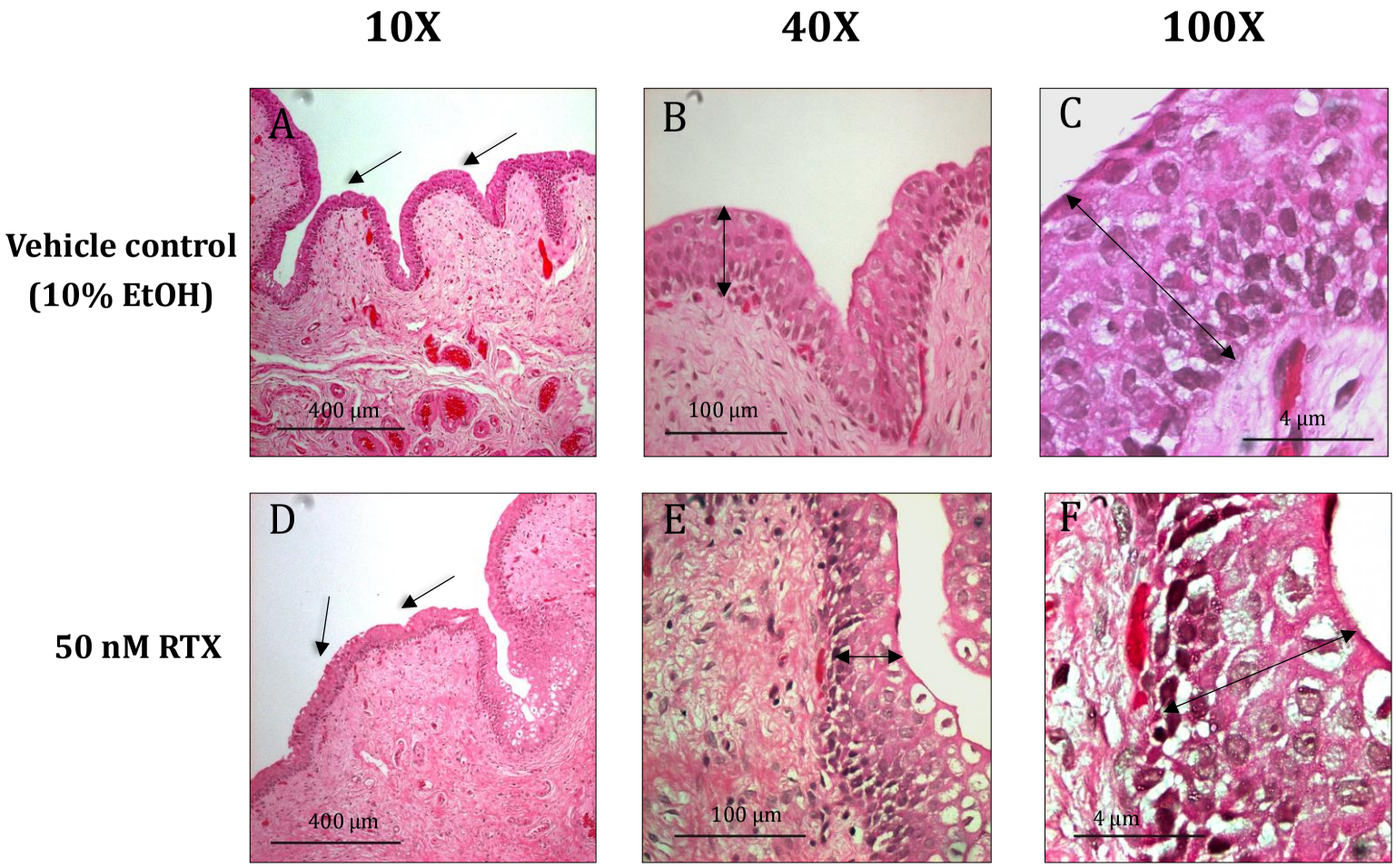

Figure 5.4: Histological sections of intact pig bladder tissue (H&E stain) with a focus on urothelium/lamina propria after luminal pre-treatment with the vehicle control for RTX (10% EtOH) and 50 nM RTX. A,B & C represent the vehicle control pre-treated tissue at 10,40 and 100x magnification. D,E & F represent tissues pre-treated with 50 nM RTX at 10,40 and 100x magnification. Black arrows indicate the thickness of the urothelium.

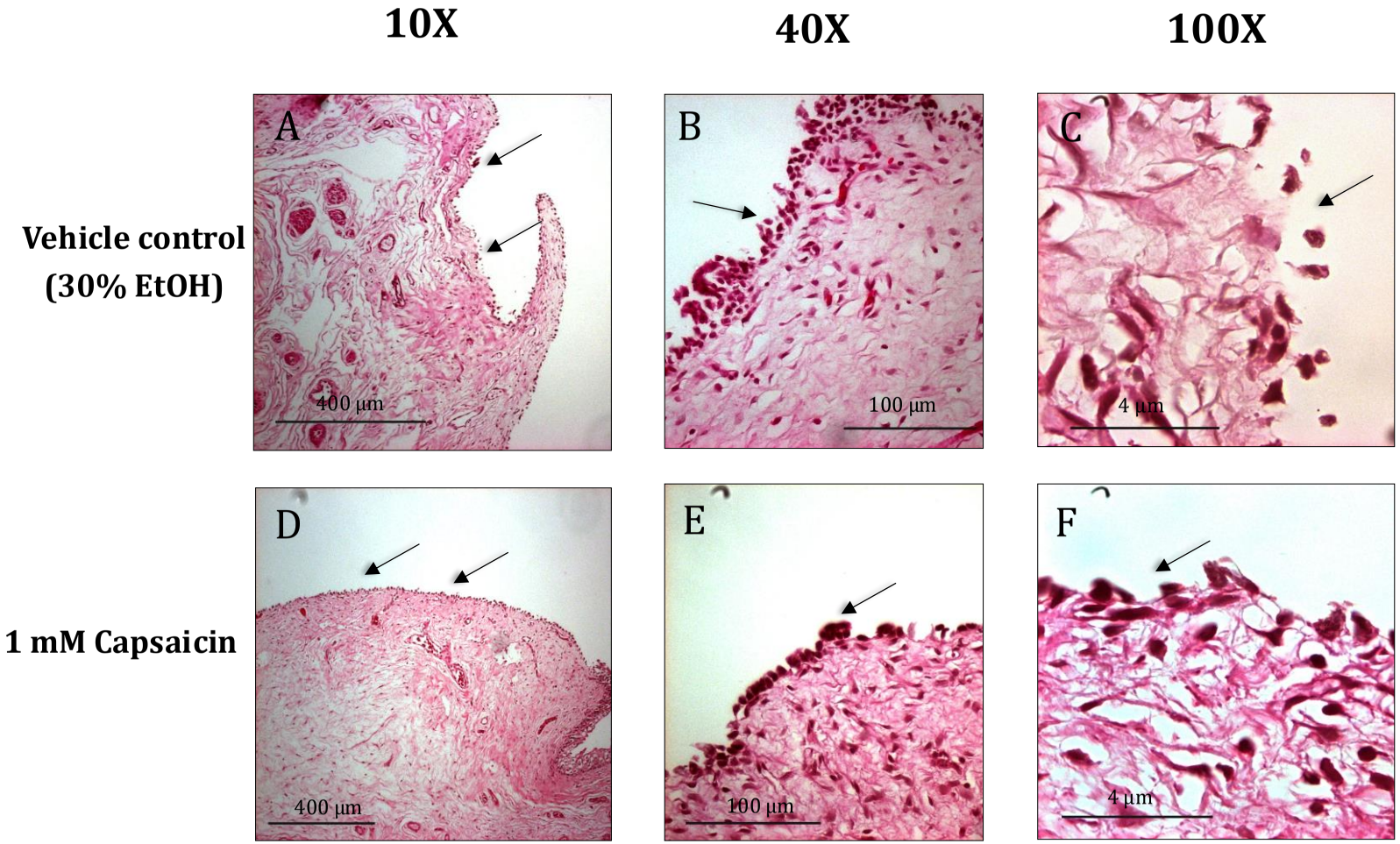

Figure 5.5: Histological sections of intact pig bladder tissue (H&E stain) with a focus on urothelium/lamina propria after luminal pre-treatment with the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin. A,B & C represent the vehicle control pre-treated tissue at 10,40 and 100x magnification. D,E & F represent tissues pre-treated with 1 mM capsaicin at 10,40 and 100x magnification. Black arrows indicate the thickness of the urothelium.

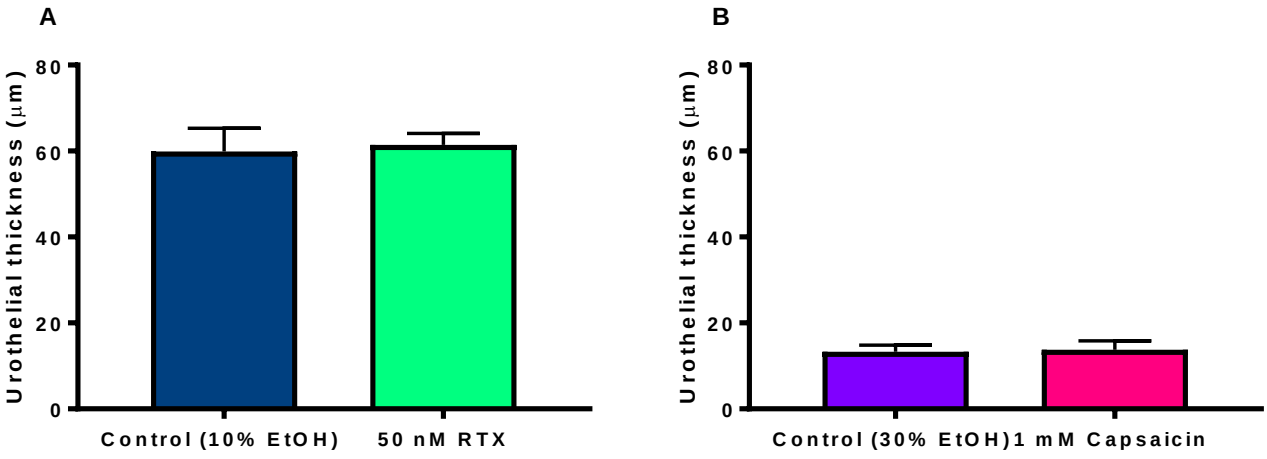

Figure 5.6: Urothelial thickness (µm) following pre-treatment with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B). Data is represented as mean ± SEM (n=6, taken from an average of 18 measurements), analysed by an unpaired two-tailed t-test.

Effects of RTX and capsaicin pre-treatment on basal and stretch-induced mediator release.

To determine the impact of luminal pre-treatment with RTX and capsaicin on the urothelium/lamina propria, the release of urothelial mediators, ATP and ACh, were examined in isolated strips of urothelium/lamina propria under basal and stretched conditions.

For tissues that were pre-treated with RTX, there was no alteration to ATP release in either basal or stretched conditions when compared to their matched vehicle controls **(Figure 5.7A)**. Although ACh could not be detected under basal conditions after pre-treatment with the vehicle for RTX (10% ethanol), ACh release remained statistically unchanged in basal or stretched conditions after pre-treatment with RTX **(Figure 5.7C)**.

Pre-treatment with capsaicin did not significantly modify ATP release in either basal or stretch conditions **(Figure 5.7B)**. Similar to pre-treating the tissue with 10% ethanol, ACh could not be detected under basal conditions after pre-treatment with ethanol vehicle for capsaicin (30% ethanol). However, pre-treating the tissue with capsaicin significantly augmented basal and stretch-induced ACh release by 6-fold and 10-fold respectively compared to their matched vehicle controls **(Figure 5.7D)**.

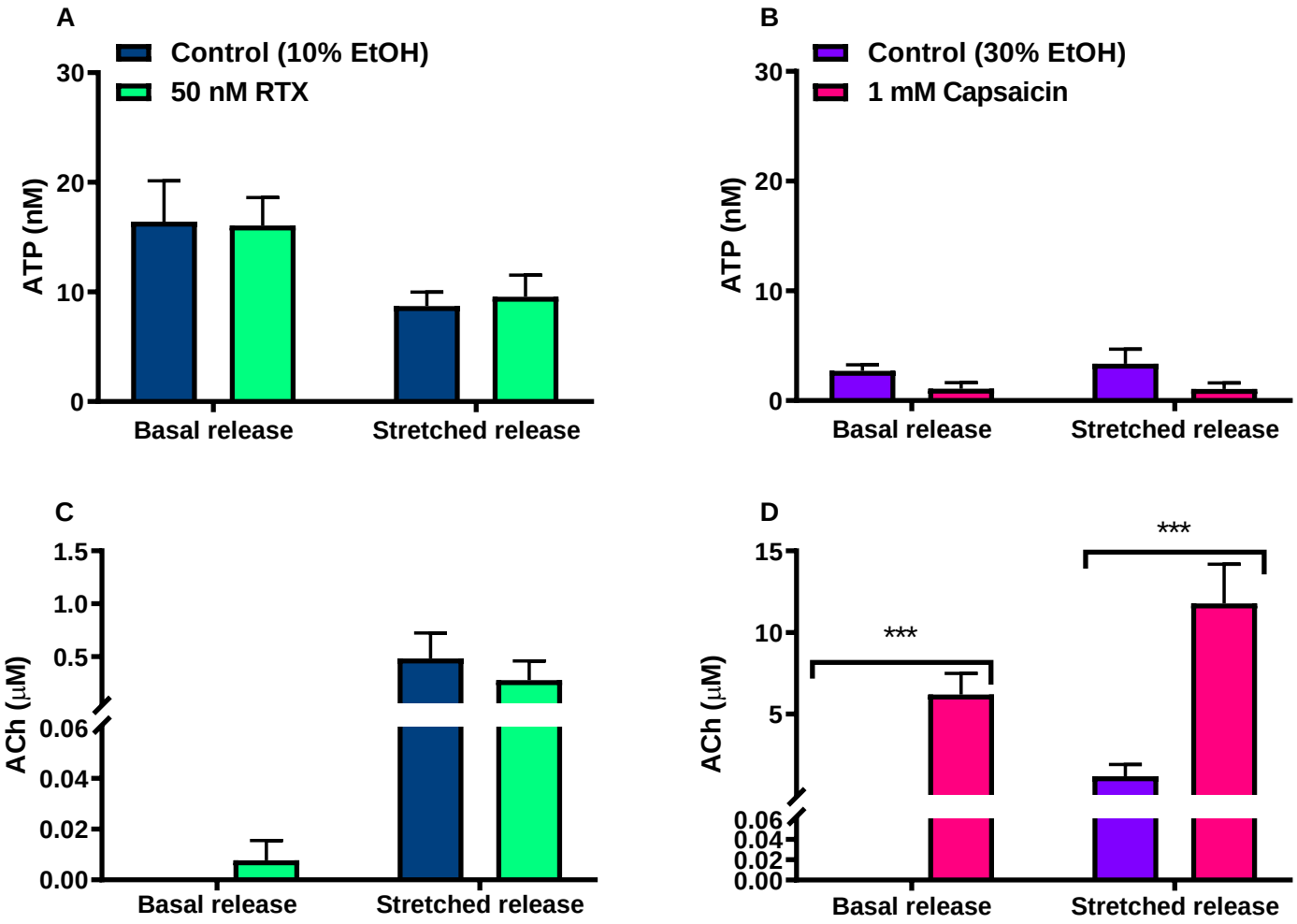

Figure 5.7: Basal and stretch-induced ATP (A&B) and ACh (C&D) release from pig urothelium/lamina propria treated with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A&C) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B&D). Data is represented as mean ± SEM (n≥7) analysed by an unpaired two-tailed t-test (*P<0.001, 30% EtOH vs. 1 mM capsaicin).**

Spontaneous contractile activity of the urothelium/lamina propria and detrusor following pre-treatment with luminal RTX and capsaicin.

Spontaneous contractile activity of urothelial/lamina propria and detrusor strips was compared between all pre-treated groups by measuring the average amplitude and frequency of spontaneous contractions. Overall, for all tissues in this study, the amplitude of detrusor spontaneous contractions was significantly lower than that found in the urothelium/lamina propria **(Figure 5.8A&B).**

For urothelium/lamina propria tissues that were pre-treated with RTX or capsaicin, there was no alteration in amplitude or frequency of spontaneous activity when compared to their matched vehicle controls **(Figure 5.8A,B,C & D)**, although the frequency of spontaneous activity in the detrusor that had been pre-treated with RTX had a tendency to be slightly depressed **(Figure 5.8C)**.

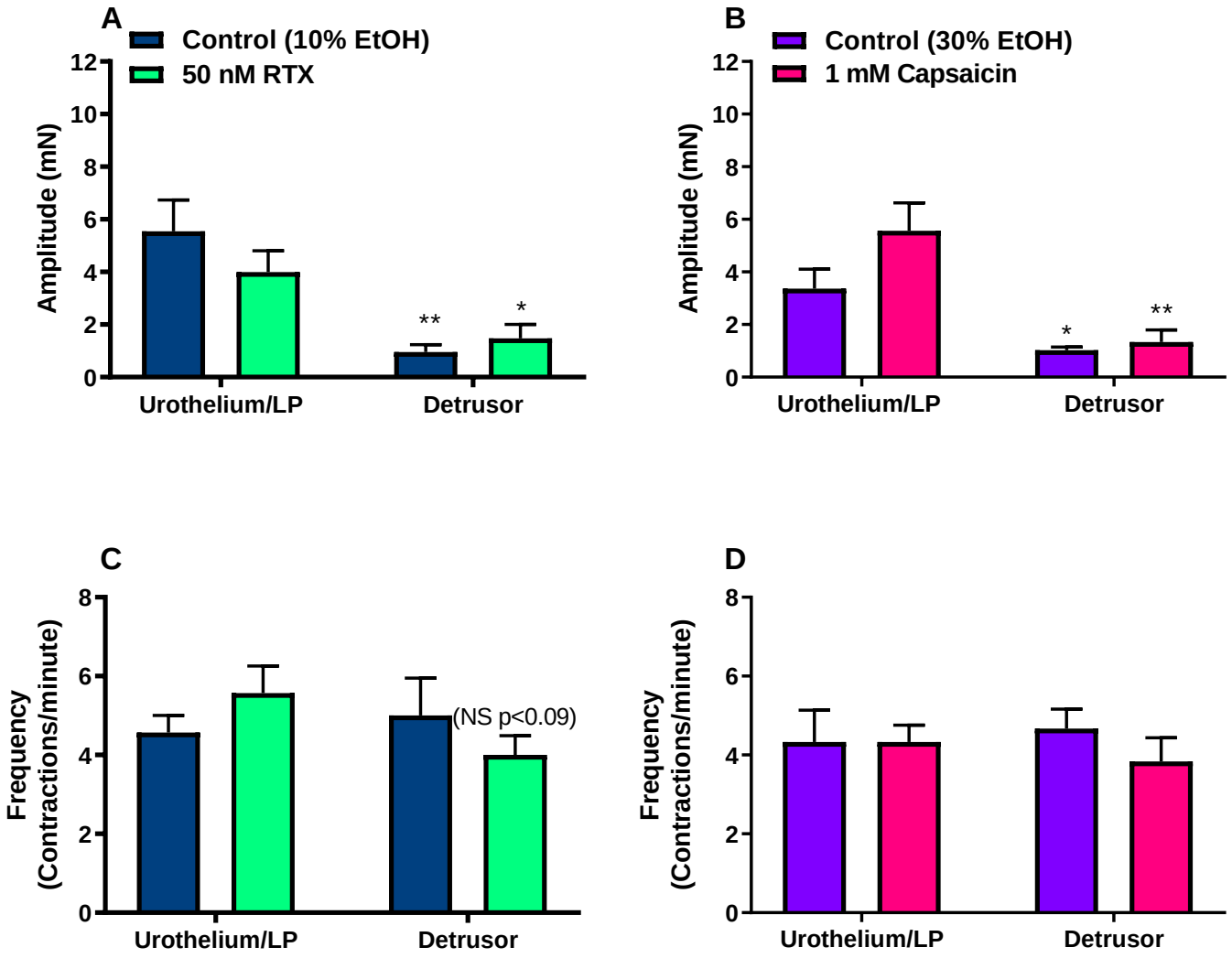

Figure 5.8: The amplitude (A&B) and frequency (C&D) of spontaneous activity in the pig urothelium/lamina propria and detrusor treated with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A&C) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B&D). Data is represented as mean ± SEM (n≥6) analysed by an unpaired two-tailed t-test (*P<0.05, **P<0.01 Urothelium/LP vs. Detrusor).

Contractile response of the urothelium/lamina propria, detrusor and intact tissue to ATP and KCl following RTX and capsaicin pre-treatment.

To investigate whether luminal pre-treatment with RTX or capsaicin had altered contractile activity to purinergic or non-receptor mediated stimulation, the responses to ATP (1mM) and KCl (60 mM) (respectively) were investigated on isolated urothelium/lamina propria, detrusor and intact tissue strips.

After pre-treatment with RTX, the urothelium/lamina propria and detrusor contractile responses to ATP remained similar to the vehicle controls **(Figure 5.9A&C)**. However, the responses of the intact tissues to ATP were significantly reduced by the same RTX pre-treatment (RTX 3.4±0.6 mN vs. 10% EtOH 8.6±1.9 mN, $P<0.05$, $n\geq7$) **(Figure 5.9E)**. The effect of RTX pre-treatment on the urothelium/lamina propria, detrusor and intact tissues to KCl stimulation remained unchanged to the vehicle controls **(Figure 5.10A,C &E)**.

Pre-treatment with capsaicin did not alter the contractile responses to either ATP **(Figure 5.9B,D &F)**, or KCl **(Figure 5.10B,D &F)**, when it was compared to the vehicle control treated tissues.

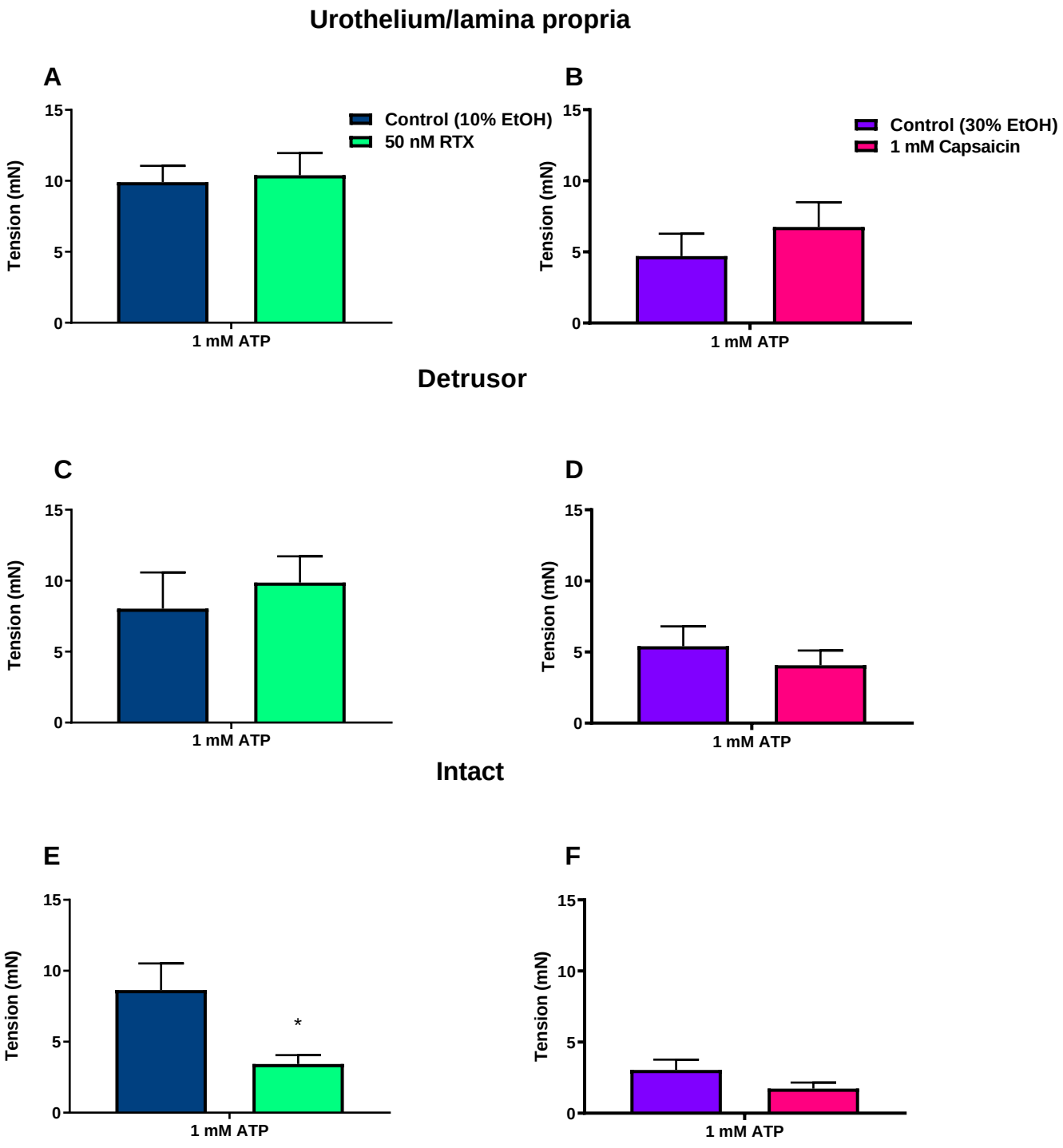

Figure 5.9: Contractile responses to 1 mM ATP of the urothelium/lamina propria (A&B), detrusor (C&D) and intact bladder tissue (E&F) that had been luminally pre-treated with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A,C&E) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B,D&F). Data is represented as mean ± SEM (n≥7) analysed by an unpaired two-tailed t-test (*P<0.05, 30% EtOH vs. 50 nM RTX).

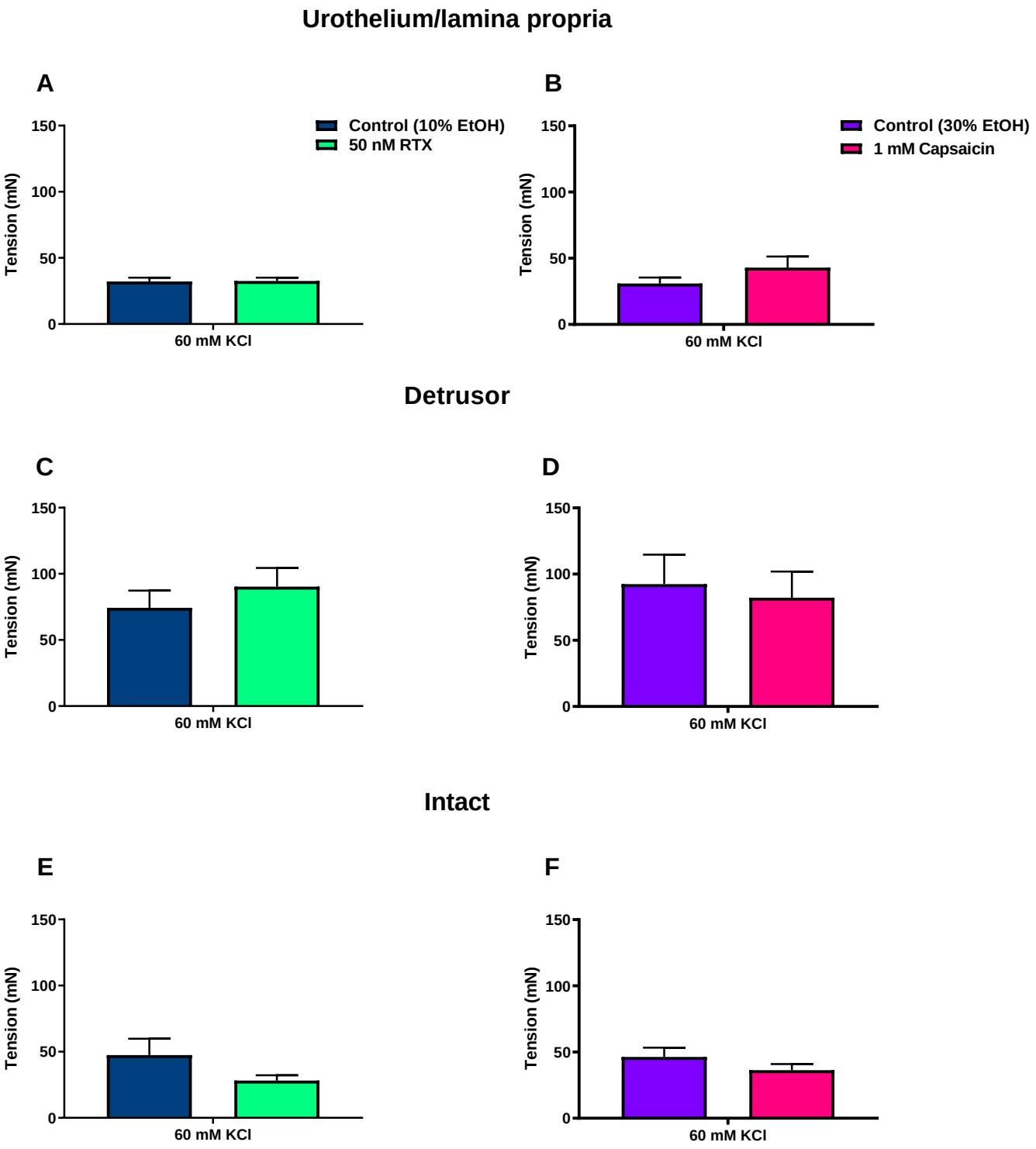

Figure 5.10: Contractile responses to 60 mM KCl of the urothelium/lamina propria (A&B), detrusor (C&D) and intact bladder tissue (E&F) that has been luminally pre-treated with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A,C&E) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B,D&F). Data is represented as mean ± SEM (n=8) analysed by an unpaired two-tailed t-test.

Contraction and relaxation responses to pharmacological agents following RTX and capsaicin pre-treatment.

Concentration-response curves to carbachol assessed the muscarinic responses in urothelium/lamina propria, detrusor and intact tissue strips while concentration-response curves to isoprenaline assessed the relaxation responses of the detrusor to determine the effects of luminal pre-treatment with RTX or capsaicin.

Cumulative concentration-response curves to carbachol were similar in RTX pre-treated and vehicle pre-treated tissues, and neither pEC_{50} nor maximum contractile responses were altered by RTX **(Figure 5.11A,C & E, Table 5.1)**.

In contrast, capsaicin pre-treatment did alter tissues responses to carbachol. Maximum responses of the urothelium/lamina propria were enhanced by 20% without any change in pEC_{50} values **(Figure 5.11B, Table 5.1)**. The detrusor responses were significantly depressed by 30% with no change to pEC_{50} values while intact bladder strips remained not significantly changed by capsaicin when compared to the vehicle **(Figure 5.11D&F, Table 5.1)**. The inhibitory effect of the urothelium/lamina propria on detrusor to maximum carbachol stimulation reduced the response by 45% after pre-treating the tissue with capsaicin in contrast to 70% inhibition by the vehicle.

For tissues that had been pre-treated with RTX or capsaicin, relaxation of the detrusor to cumulative concentrations of isoprenaline was not statistically different to the vehicle controls with no change to the pEC_{50} values **(Figure 5.12A&B, Table 5.1)**.

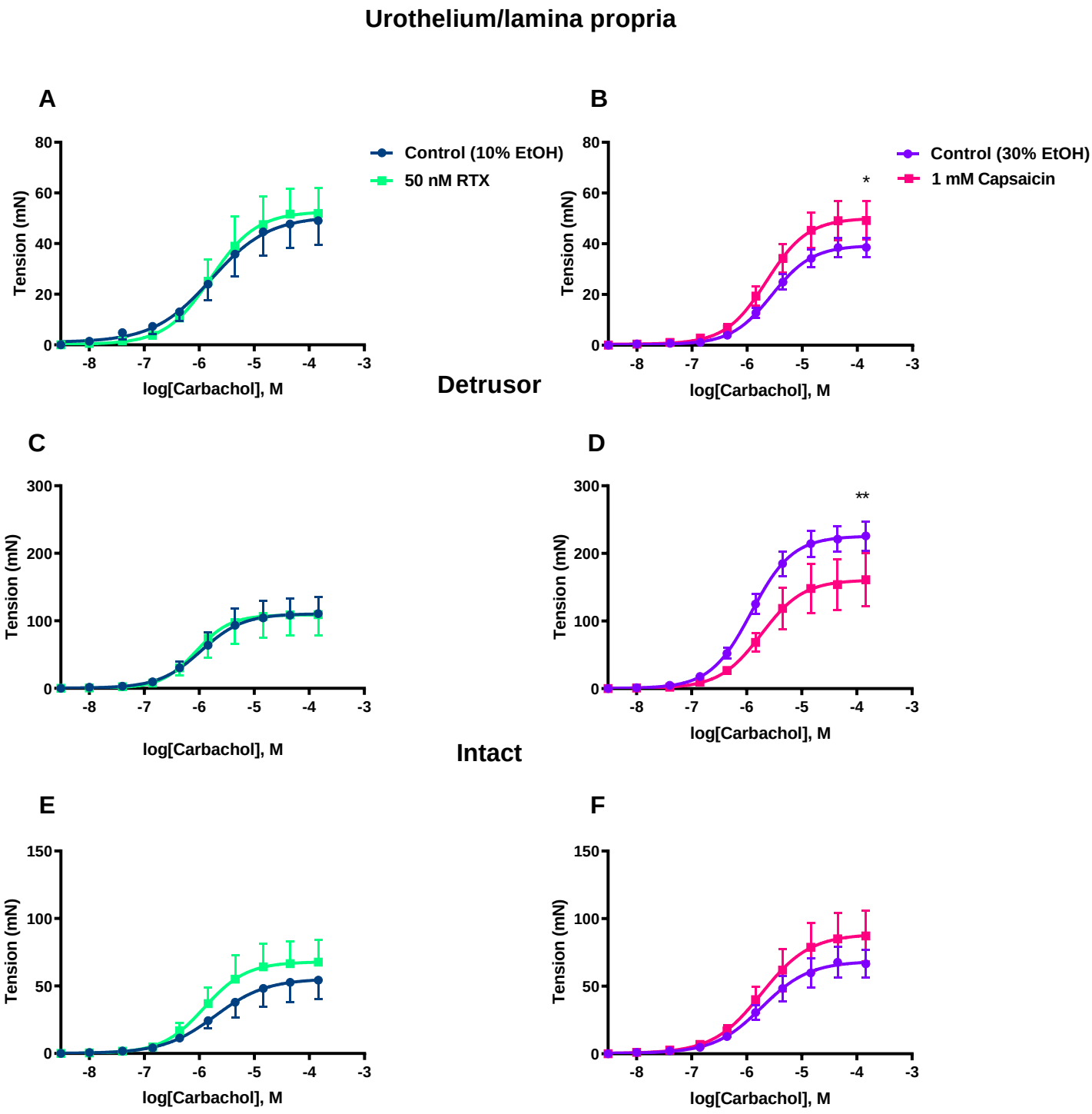

Figure 5.11: The contractile response of pig urothelium/lamina propria (A&B), detrusor (C&D) and intact tissue (E&F) following luminal pre-treated with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A,C,E) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B,D,F) to cumulative concentrations of carbachol. Data was analysed with multiple comparison F-test followed by an unpaired two-tailed t-test (n≥6), (*P<0.05, **P<0.01, 30% EtOH vs. 1 mM capsaicin).

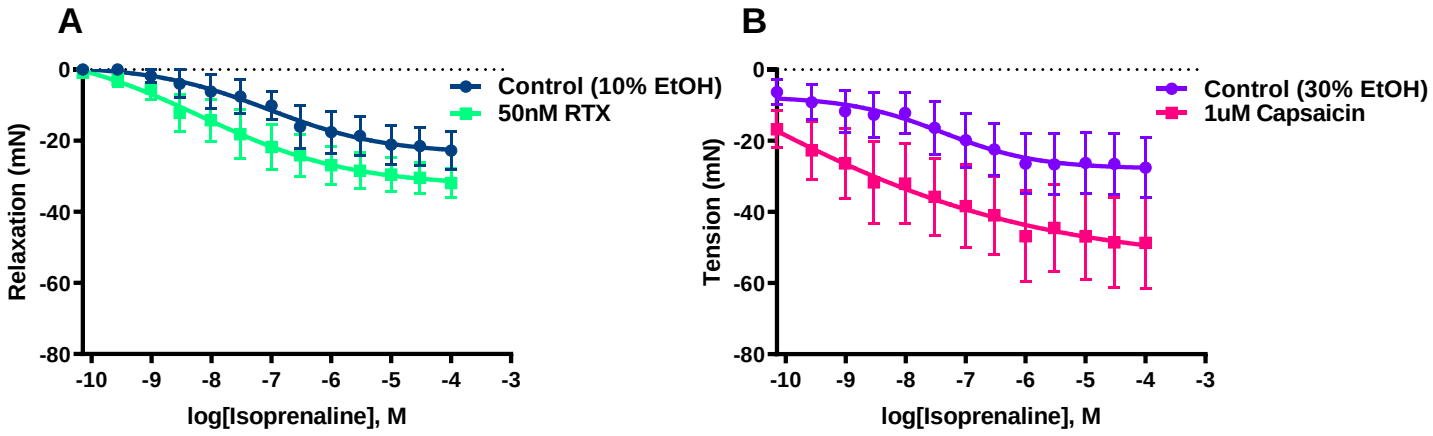

Figure 5.12: The relaxation response of the detrusor following luminal pre-treatment with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A) or the vehicle control for (30% EtOH) and 1 mM capsaicin (B) to cumulative concentrations of isoprenaline. Data was analysed with multiple comparison F-test followed by an unpaired two-tailed t-test (n≥7).

Table 5.1: Mean (± SEM), maximum responses and pEC_{50} values for carbachol and isoprenaline. Data was analysed with multiple comparison F-test followed by an unpaired two-tailed t-test (n≥6), (*P<0.05, **P<0.01, 30% EtOH vs. 1 mM capsaicin).

	Control (10%)	50 nM RTX	Control (30%)	1 mM capsaicin
Carbachol	**Urothelium/Lamina Propria**			
pEC_{50} (± SEM)	5.79± 0.26	5.79 ± 0.22	5.55 ± 0.08	5.65 ± 0.13
Maximum response (mN)	50.78 ± 6.62	52.66 ± 5.96	39.34 ± 1.99	50.12 ± 3.74 *
	Detrusor			
pEC_{50} (± SEM)	5.97 ± 0.22	6.06 ± 0.24	5.92 ± 0.08	5.74 ± 0.2
Maximum response (mN)	110.5 ± 11.68	108.5 ± 12.92	225.7 ± 9.4	160.8 ± 17.78 **
	Intact			
pEC_{50} (± SEM)	5.72 ± 0.28	5.91 ± 0.24	5.73 ± 0.16	5.74 ± 0.22
Maximum response (mN)	55.37 ± 8.21	67.92 ± 8.22	68.45 ± 5.97	88.61 ± 10.38
Isoprenaline	**Detrusor**			
pEC_{50} (± SEM)	7.04 ± 0.65	8.08 ± 1.17	7.38 ± 0.95	11.08 ± 39.47
Maximum response (mN)	-23.61 ± 5.53	-32.7 ± 6.09	-27.84 ± 5.51	-54.92 ± 50.95

Contractile responses of the detrusor to electrical field stimulation following RTX and capsaicin pre-treatment.

Electrical field stimulation was used to investigate any changes to parasympathetic nerve-mediated contraction of the detrusor that may have occurred after luminal pre-treatment with either RTX or capsaicin.

For tissues that were pre-treated with RTX, there was no change to the contractile responses of the detrusor to EFS at all frequencies tested when compared to the vehicle controls **(Figure 5.13A)**. In the presence of the muscarinic antagonist atropine (1 µM), the reduced responses to EFS were similar to the reductions found in vehicle controls. After desensitization of the P2X receptors with α,β m-ATP (10 µM), the detrusor responses to EFS after RTX pre-treatment were not altered any further nor were they different to the vehicle controls**(Figure 5.13C&E)**. This shows that ACh is the main neurotransmitter in the detrusor and is unaltered after pre-treatment with RTX.

Likewise, for tissues pre-treated with capsaicin, there was also no change to the contractile responses of the detrusor to EFS at all frequencies when compared to the vehicle **(Figure 5.13B)**. The reduced responses to EFS in the presence of atropine was also comparable to the vehicle which also remained unaltered after desensitization of the P2X receptors with α,β -mATP and similar to the vehicle controls **(Figure 5.13D&F)**. This indicates that ACh is the main neurotransmitter in the detrusor after pre-treatment with capsaicin.

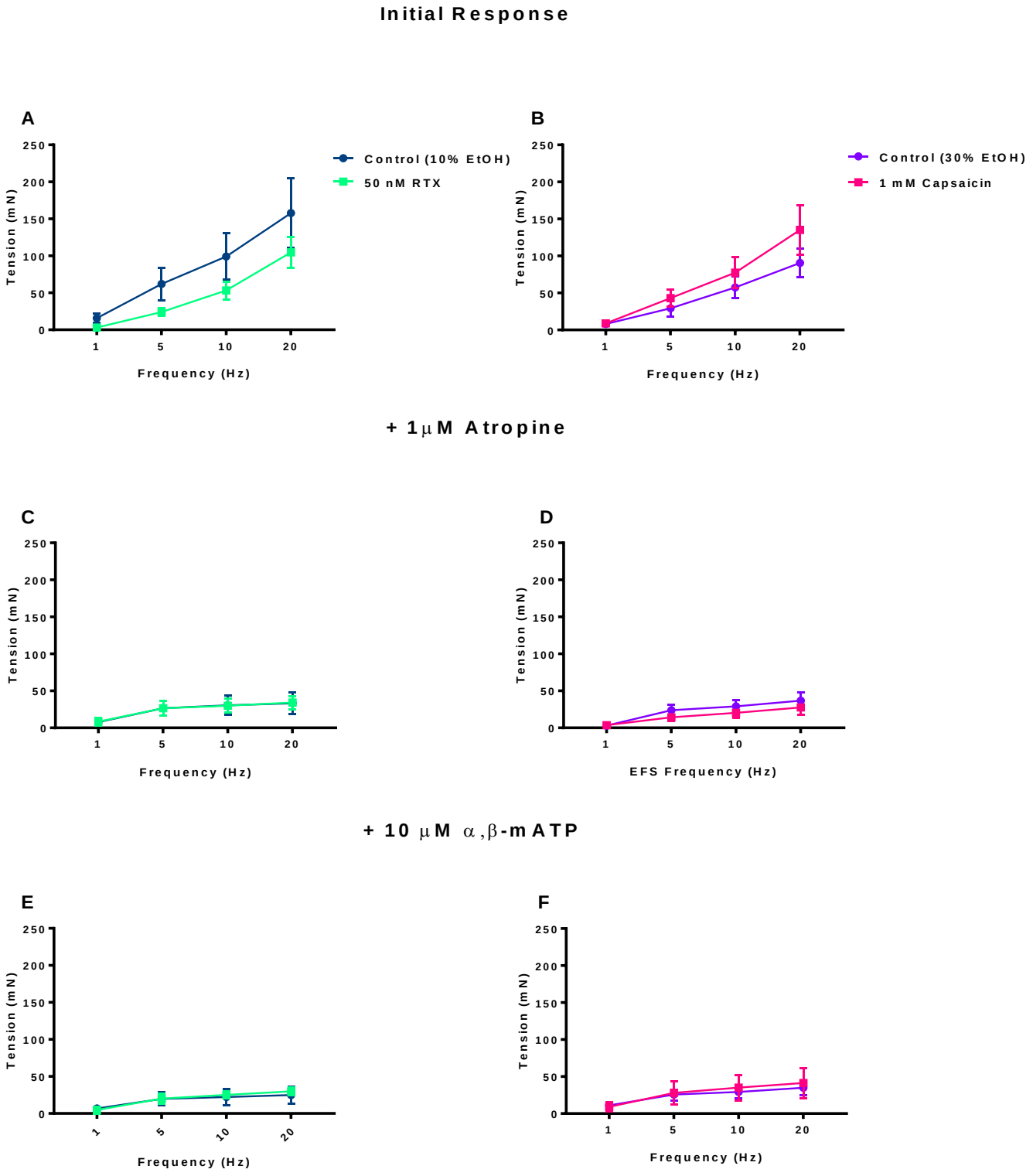

Figure 5.13: Responses to EFS (20v, 1ms pulse width, 5s train) of pig detrusor tissue luminally pre-treated with the vehicle control for RTX (10% EtOH) and 50 nM RTX (A,C&E) or the vehicle control for capsaicin (30% EtOH) and 1 mM capsaicin (B,D&F) in the presence of 1 µM atropine (C&D) and 10 µM α,β –mATP (E&F). Data is represented as mean ± SEM (n≥5) analysed by an unpaired two-tailed t-test.

5.5 Discussion

By the end of the last chapter, it was concluded that 10% ethanol (the vehicle for RTX) had not penetrated any further than the basal layer of cells in the urothelium in contrast to 30% ethanol (the vehicle for capsaicin). However, it was ascertained that both the ethanol treatments had overcome the urinary GAG layer which protects the underlying structures from noxious compounds which may potentially allow for drug diffusion to the deeper layers. Both RTX and capsaicin are much larger molecules than ethanol. RTX has a molecular weight of 628.72 while capsaicin has a molecular weight of 305.41 and both these molecules are highly lipophilic suggesting that they can diffuse through the plasma membranes and the intercellular spaces (Mishina et al., 1986, Appendino and Szallasi, 1997, Jung et al., 1999). Activation of TRPV1 channels on TRPV1 positive cells by RTX and capsaicin leads to the influx of Ca^{2+} and Na^{+} in a dose-dependent manner which promotes depolarization, desensitization to further noxious stimuli and neurotoxicity in DRG neurons (Wood et al., 1988, Winter et al., 1990, Acs et al., 1996). However, desensitization to RTX and capsaicin does not appear to occur in urothelial cells (Birder et al., 2001). RTX is more potent than capsaicin, desensitizing bladder tissue at nanomolar concentrations and due to their desensitizing capabilities, these compounds in theory, could be useful for treating refractory IC/BPS where altered sensory nerve functioning may play a role (Maggi et al., 1990b, Pang et al., 1995).

As RTX and capsaicin are not approved for routine clinical use for the treatment of IC/BPS, varying concentrations have been used in experimental conditions. The concentrations for RTX have not been clearly defined yet and can range from 1 nM-1 µM with spurious results. While some improvements have been achieved with a single instillation of 10 nM in the bladder, 50 nM was chosen for this study as it is more frequently described in clinical studies in the literature (Chancellor and de Groat, 1999, Lazzeri et al., 2000, Chen et al., 2005b, Payne et al., 2005). The most commonly used concentration of capsaicin that has been used clinically for other bladder pathology such as overactive bladder is 1 or 2 mM. As promising results for hypersensitive bladders have been achieved with 1 mM of capsaicin, this concentration was selected for this study (Cruz et al., 1997, Chancellor and de Groat, 1999).

The effect of luminal RTX and capsaicin on the urothelium/lamina propria during/post-treatment.

Identical to the format from the previous chapter, this study looked at the immediate interaction that luminally applied RTX or capsaicin had while it was in contact with the urothelium.

RTX

During treatment with RTX, there were no additional alterations to ATP, ACh release and LDH activity. Adenosine 5'-triphosphate release during treatment had already been enhanced by the vehicle for RTX (10% ethanol) and therefore remains elevated (see preceding chapter). These results indicate that at this concentration, RTX is unlikely to have created any further damage. This is supported by Farfariello et al. (2014) who found that RTX at concentrations of >19.9 µM was cytotoxic to T24 and 5637 bladder cancer cell lines, which were more sensitive to RTX than normal human urothelial cell lines.

Histological evidence agrees with this finding as the thickness of the urothelium that had been pre-treated with RTX remained unchanged compared to its vehicle. Although macroscopically, some structures in the basal urothelial layer appeared swollen. However, any alterations that may occur are not long-term as human bladders that have been treated with 50 nM RTX (including ethanol vehicle) found no significant histological alterations or denudation of the urothelial layer many months after instillation (Silva et al., 2001).

Capsaicin

During treatment with capsaicin, ATP release was similar to that of its vehicle (30% ethanol) which was already enhanced. Although the role of TRPV1 channels in the urothelium is controversial, Charrua et al. (2009) and Birder et al. (2002) found that stimulation of the TRPV1 channels on human urothelial cells and rat urothelial cells with capsaicin (1 µM and 100 nM respectively) evoked ATP release while capsazepine (TRPV1 antagonist) blocked this response confirming that this receptor was responsible. For those experiments, it was not clear which solvent was used to dissolve the capsaicin or whether the vehicle was even used in control experiments. In contrast, studies on cultured pig urothelial cells and urothelial/lamina propria preparations found no ATP release in response to 0.1-10 µM capsaicin. However, the solvent used to prepare capsaicin for these experiments was DMSO and Tween-80 and ethanol

in very low concentrations (Sadananda et al., 2012). We have previously shown (chapter 4) that ethanol evokes ATP release while in contact with the urothelium/lamina propria which is likely to be responsible for the enhanced release found in the present study.

With regards to ACh release during treatment, capsaicin on the urothelial/lamina propria produced a seventeen-fold greater increase in release which usually is depressed by its vehicle. It is unclear where ACh would be originating from given the destruction of the urothelial cell layer, but a contributing factor may be related to the desensitization process (Maggi et al., 1990b). To clarify, the sloughing effect of the vehicle would allow capsaicin to diffuse without difficulty through the layers of the bladder to reach its target TRPV1 channels found on sensory nerve fibers in the sub-urothelial layer (Ost et al., 2002). Capsaicin's action on the bladder sensory neuron terminals promotes the liberation of neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) that are suspected to play a role in its initial stimulatory activity (Maggi et al., 1987c, Maggi et al., 1990b, Szallasi, 1996). In particular, SP has been shown to evoke ACh release in a concentration-dependent manner in guinea pig bladder strips (Shirakawa et al., 1989). It is therefore proposed that this mechanism may be promoting the enhanced mediator levels. In support, studies in guinea pig ileum tissue have also identified that the initial contractile response to capsaicin is mediated in part by SP and ACh while SP activates cholinergic neurons in the longitudinal muscle of this tissue (Bartho and Szolcsanyi, 1978, Holzer and Lembeck, 1980, Bartho et al., 1982). An additional contributor to this enhanced release could also be the inhibition of acetylcholinesterase activity since Orhan et al. (2007) found that a synthetic capsaicin analogue potently inhibited this activity.

The LDH activity during treatment with capsaicin was similar to the vehicle. Therefore, it seems unlikely that capsaicin has caused any further damage to the urothelium/lamina propria even though a histological analysis by Byrne et al. (1998) observed more pronounced damage compared to 30% ethanol alone in the rat bladder. Furthermore, rat lung tissue exposed to aerosols of capsaicin (1-1.2 mg/kg) and ethanol for 30-minutes produced a variety of lesions along with haemorrhage and congestion while the control animals exposed to ethanol vapours had no lesions (Reilly et al., 2003). The histological analysis of the current study supports that no further damage to the urothelium/lamina propria has occurred as a result of pre-treatment with capsaicin. However, bear in mind that urothelial thickness was already reduced by approximately 75% after pre-treatment with the ethanol vehicle.

The urothelium/lamina propria function following pre-treatment

In line with chapter four, it was important to assess urothelial/lamina propria function after luminal pre-treatment. Accordingly, ATP and ACh release were examined in isolated urothelial/lamina propria strips under basal and stretched conditions. Generally, non-neuronal ATP and ACh release is enhanced by the stretch of urothelial tissues. However, as mentioned in the previous chapter, ATP release from the urothelium/lamina propria can be spontaneous and occur in cyclical surges that can significantly affect the concentration, let alone any interference that treatment may have (Kumar et al., 2004, Yoshida et al., 2006, Sui et al., 2014).

RTX

Curiously, for tissues that had been pre-treated with the vehicle (10% ethanol), ATP release found in both basal and stretched conditions was more than double of that found in the previous chapter when 10% ethanol was compared 0.9% saline (control)**.** The disparity in ATP levels is likely due to cyclical ATP surges that have been found from the same tissue in other studies (Sui et al., 2014, Kushida and Fry, 2016). Pre-treatment with RTX produced ATP and ACh releases that were similar to the vehicle controls in both basal and stretched conditions.

Capsaicin

For urothelial/lamina propria tissue strips pre-treated with capsaicin, ATP release was similar to the vehicle control release which is ordinarily depressed in basal and stretched conditions. The low levels of ATP release along with the absence of the urothelial cell layer confirm the findings made by others that the primary source of ATP in the bladder originates from the urothelium (Kumar et al., 2004, Sui et al., 2014).

Remarkably for these experiments after pre-treatment with capsaicin, there were increases in ACh release that were six and ten-fold greater in both basal and stretch conditions respectively when they were compared to the vehicle which had undetected basal releases. The enhancement of ACh release is similar to what was found during treatment and may be related to the action of capsaicin liberating neuropeptides from the sensory nerves such as SP that has been found to elicit the release of ACh from bladder strips (Shirakawa et al., 1989, Maggi et al., 1990b). This is in addition to the inhibited acetylcholinesterase activity ascribed to capsaicin preventing its degradation (Orhan et al., 2007). It is unlikely the increase in ACh

release is from capsaicin-mediated damage to cholinergic nerve terminals promoting its seepage into the surrounding tissues as capsaicin has been known not to affect cholinergic or adrenergic motor nerves. (Gamse et al., 1982, Holzer, 1991).

The effect of luminal RTX and capsaicin on bladder contractile/relaxation mechanisms following pre-treatment

The results so far suggest that RTX has not impacted the functioning of the urothelium/lamina propria in any way. This is consistent with the reported actions of RTX generally favouring desensitization rather than excitation. Capsaicin, on the other hand, has increased the urothelial release of ACh during and after luminal treatment, these effects being in addition to the alterations caused by the ethanol. It is not known how deeply into the tissue RTX or capsaicin can penetrate, therefore the contractile/relaxation functions of the urothelium/lamina propria, detrusor and intact strips were examined and compared to their respective vehicles.

Spontaneous activity of the urothelium/lamina propria and detrusor

The mechanisms behind spontaneous activity in the bladder are unclear. Complex interactions involving ATP, ACh and prostaglandins are thought to contribute to this activity. (Maggi, 1992, Moro et al., 2011, Nile and Gillespie, 2012, Kobayter et al., 2012, Kushida and Fry, 2016) Spontaneous activity has also been shown to be more pronounced in urothelium/lamina propria preparations when compared to the detrusor (Kushida and Fry, 2016). For this study, all urothelium/lamina propria tissues had higher contractile amplitudes compared to their matching detrusor. Pre-treatment with RTX did not affect the amplitude or frequency of spontaneous activity beyond the vehicle in which amplitude is normally enhanced. Also, pre-treatment with capsaicin was of no consequence on spontaneous contractile amplitude or frequency.

The contractile responses to ATP and KCl, muscarinic and parasympathetic stimulation (EFS)

Following pre-treatment with RTX or capsaicin, the urothelium/lamina propria, detrusor and intact tissue strips all contracted in response to ATP, KCl, muscarinic and parasympathetic stimulation. The specific changes as a result of treatment are discussed below.

RTX

The response of the urothelium/lamina propria and detrusor to purinergic stimulation remained unchanged following pre-treatment with RTX. However, the response of the intact tissue was depressed by 60%. As there was no change to the isolated urothelium/lamina propria and detrusor responses for this experiment, for the intact tissue, the relay of purinergic signals from the urothelium/lamina propria to the underlying detrusor may have been altered by pre-treatment with RTX (Parsons et al., 2012). This may reflect the properties of RTX reported to have a much slower onset of action which favours desensitization as opposed to the initial excitation produced by capsaicin (Winter et al., 1990, Maggi et al., 1990b). In the whole rat bladder, Maggi et al. (1990b) found that low concentrations of RTX reduced the contractile effect of subsequent applications of capsaicin when it was applied 60-minutes later.

Moreover, in cultured rat urothelial cells, 100 nM of RTX increased NO release (Birder et al., 2001). While in the pig bladder, Moro et al. (2012) reported that nitric oxide synthase (NOS) inhibitor L-NNA enhanced the detrusor responses to EFS. Thus, altered transfer of purinergic signalling from the urothelium to the detrusor and an increase in NO activity may also be reducing the intact tissue responses to RTX in the pig bladder.

The response of all tissue strips pre-treated with RTX to KCl and muscarinic stimulation remained unchanged to the control values. Also, the response of the detrusor to EFS after luminal pre-treatment with RTX produced no additional alterations to the contractile response. Similarly, Maggi et al. (1990b) identified that bladder treatment with 10 nM RTX did not significantly affect the amplitude of distension evoked micturition contractions in rat bladders although volume threshold increased. In the current study, the contributions of ACh and ATP to nerve-mediated responses in the detrusor to EFS remained unchanged to the vehicle demonstrating that ACh is the dominant neurotransmitter after pre-treatment with RTX. Overall, pre-treatment with RTX does not change parasympathetic nerve activity and cholinergic responses. However, the purinergic responses of the intact tissues are depressed.

Capsaicin

Purinergic stimulation and depolarization with KCl did not alter the responses of the urothelium/lamina propria, detrusor and intact tissues pre-treated with capsaicin beyond that of the ethanol vehicle. Although it should be noted that the responses of the urothelium/lamina propria pre-treated with the ethanol vehicle compared to saline controls to purinergic stimulation were depressed while the responses of the same tissue to KCl were enhanced.

Muscarinic stimulation enhanced the contractile response of the urothelium/lamina propria by a further 20% in addition to the enhancements made by the vehicle. The increase in response may be due to capsaicin desensitizing and depleting the sub-urothelial afferent nerves, the enhanced levels of ACh found in the urothelium/lamina propria and the general reactivity of the tissues exposed to noxious agents such as K^+ found in the bathing medium due to the ruptured urothelium (Maggi et al., 1989a, Parsons et al., 1994, Smet et al., 1997, Moro et al., 2012). Additionally, It has been found in cultured dorsal root ganglion neurons of the rat that high levels of 3'-5'- cyclic adenosine monophosphate (cAMP) opposed capsaicin depolarization and desensitization (Matsushita et al., 2018). As ethanol has been found to enhance cAMP, the ethanol vehicle for these experiments may be hindering desensitization and prolonging the process (Hoffman and Tabakoff, 1990). Interestingly, the inhibitory effect that the urothelium/lamina propria has on the detrusor was reduced by 25%, most likely due to the extensive damage of the urothelial layer reducing the expression of UDIF (Hawthorn et al., 2000)

For the detrusor pre-treated with capsaicin, the response to muscarinic stimulation was depressed by approximately 30% compared to the vehicle which normally enhances the response. Detrusor muscle relaxation is not uncommon in the presence of capsaicin (Maggi et al., 1987b, Tucci et al., 2002). It was found in lamb detrusor strips that indomethacin (COX inhibitor) prevented the initial contraction and strongly inhibited capsaicin-induced relaxation while tissue was maintained at a passive tension of 4 g in organ baths. Atropine in the same study inhibited the initial contractile response but did not affect the relaxation response. The authors suggest that capsaicin directly simulated prostaglandin production which acts on the nerves releasing ACh and CGRP as CGRP antagonist also prevented capsaicin-induced relaxation (Tucci et al., 2002). GGRP relaxes the detrusor in some species but not in humans and rats (Maggi, 1995b) It is unlikely that CGRP is mediating the relaxation response in our

study as it has been found in pig detrusor that CGRP had no effect on the contractions induced by K+, carbachol, SP and EFS (Persson et al., 1991). Other suggested peptides that may contribute to the relaxation response in the detrusor is vasoactive intestinal peptide which has been found to attenuate responses to carbachol in the pig detrusor. (Klarskov et al., 1984, Lepiarczyk et al., 2017) Other explanations for the reduction in contractile amplitude to carbachol could be the non-specific effects of capsaicin described by Maggi et al. (1989b) which can take up to 15-hours to be restored (Cheng et al., 1993).

No additional effects to muscarinic stimulation were observed in the intact tissues pre-treated with capsaicin. This is in-line with the findings of Maggi et al. (1984) who observed no difference in amplitude produced by the topical application of acetylcholine onto whole rat bladders that had been desensitized by capsaicin.

While the detrusor responses were depressed by muscarinic stimulation, this produced no further changes to the response to EFS for all frequencies tested when it was compared to the vehicle. Although, the vehicle alone enhances the responses to maximum stimulation. Pre-treatment with capsaicin does not affect the contribution of ACh or ATP mediating the parasympathetic response in the detrusor as blocking the muscarinic receptors reduced most of the contractile response to EFS in which no further changes occurred by desensitizing the purinergic receptors. Furthermore, it was observed in rat bladders desensitized to 1-3 μM of capsaicin that the amplitude of contractions elicited by EFS was unchanged 1-3 hours after its application (Santicioli et al., 1986). On the whole, pre-treatment with capsaicin altered parasympathetic cholinergic responses. These effects are in addition to those produced by the vehicle.

The relaxation response of the detrusor to β-adrenoceptor stimulation with isoprenaline

There were also no additional alterations to detrusor relaxation in response to β-adrenoceptor stimulation after pre-treatment with either RTX or capsaicin indicating that these treatments have not impacted sympathetic activity.

Implications for IC/BPS

RTX

RTX seems theoretically attractive to treat IC/BPS with its ability to desensitize the upregulated sub-urothelial TRPV1 positive nerve fibres without prior excitation (depending on concentration) and its less severe side effects compared to capsaicin (de Seze et al., 1998, Lazzeri et al., 2000, Chen et al., 2005b, Payne et al., 2005, Apostolidis et al., 2006, Liu et al., 2014). However, conflicting clinical results do not support its use. It is also uncertain how far RTX (using 10% ethanol as a vehicle) would penetrate the IC/BPS bladder which would appear to be dependent on disease progression and the state of the urothelium (Tomaszewski et al., 2001, Parsons, 2011). There is very little evidence in this study that 50 nM RTX has reached its sub-urothelial target in the pig bladder. Some of this uncertainty has also arisen from the findings of the last chapter regarding the effectiveness of the vehicle for RTX (10% ethanol) to penetrate far enough for RTX to reach its target. The reduced response of the intact tissue to purinergic stimulation was speculated to be RTX interference with urothelium/lamina propria altering the signalling to the underlying detrusor as there were no changes to the contractile response of the detrusor to ATP. Despite this, RTX may have a much slower diffusion and onset through the urothelium as intravesical studies in rats found that RTX (>100 nM) diffusion through the urothelium/lamina propria was very slow that impacted the arterial pressor responses during bladder contraction four days after treatment (Chuang et al., 2001). The concentration of RTX may also be a factor, in the same study Chuang et al. (2001) noted no changes to cystometrogram parameters until RTX concentrations had reached 100 nM.

Furthermore, pig bladders that had been intravesically treated with RTX (500 nM) found modifications to cholinergic and noradrenergic nerve fibres in the trigone region seven days after treatment (Lepiarczyk et al., 2017). Assuming RTX can penetrate the urothelium far enough to reach the afferent fibres, it has also been identified in electrophysiological experiments that the RTX induced current on rat DRG neurons had a much slower onset that was persisting when compared to capsaicin while the down-regulation of binding sites in the bladder took 24-hours to develop (Winter et al., 1990, Goso et al., 1993). A slower onset of desensitization may be of benefit to patients with sensitive bladders. However, for humans and pigs there is evidence that RTX binds to TRPV1 channels with a lower affinity when compared to the rat, although, interestingly, some neuroleptic drugs such as rimcazole can facilitate RTX binding to human TRPV1 channels (Szallasi and Blumberg, 1990, Acs et al., 1994, Acs et al.,

1995). Other factors that have been found to influence RTX binding is NGF. It has been found in rat DRG cultures that the absence of NGF leads to loss of RTX binding sites (Winter et al., 1993). Assuming RTX is able to diffuse to the afferent nerves using its current vehicle, it is uncertain whether the elevated levels that have been found in IC/BPS patients would enhance binding to its receptor (TRPV1) (Lowe et al., 1997, Liu and Kuo, 2007b). Another consideration is the interaction RTX has with ethanol on the bladder tissues, on one hand, ethanol potentiates the Ca^{2+} response to capsaicin at the TRPV1 channel, on the other hand as ethanol has been found to enhance cAMP and adenosine levels in various cells and tissues (Nagy et al., 1989, Clark and Dar, 1989, Nagy et al., 1990, Hoffman and Tabakoff, 1990, Nagy, 1994, Trevisani et al., 2002). Cyclic AMP has been found to oppose capsaicin depolarization and desensitization while adenosine has been found to inhibit activation of the TRPV1 receptor (Puntambekar et al., 2004, Matsushita et al., 2018).

To suggest 50 nM RTX and its combination with 10% ethanol is effective for symptom relief for BPS sufferers is unknown and beyond the scope of this study.

Capsaicin

More favourable results are apparent with the use of capsaicin, however, due to its pungency, the studies are limited. It should be noted that although patients may experience discomfort with the initial instillation, successive instillations become less intense (Lazzeri et al., 1996). We can verify that much of intolerable side effects associated with the instillation of capsaicin are instigated by its vehicle (30% ethanol). These reported effects can take up to two weeks to resolve (de Seze et al., 1998). Although there are unpleasant side effects, the use of 30% ethanol can overcome the GAG and urothelial cell layer to allow capsaicin to diffuse freely to its target TRPV1 channels on the afferent fibres found in the sub-urothelium. The additional side effects that were associated with the addition of capsaicin for this study were the elevations in ACh release from the urothelium/lamina propria that were persisting. These elevations were enhanced not only during treatment but hours after the washout of capsaicin while the urothelium/lamina propria was under basal and stretched conditions. For patients with IC/BPS this altered ACh release could be altering the regulation of other transmitters, enhancing sensations and activating the elevated M2 receptors found in the sub-urothelium (Mukerji et al., 2006b, Braverman et al., 2007, Kullmann et al., 2008b, Daly et al., 2010, Moro et al., 2011). This could also potentially limit the storage phase for the bladder (Yoshida et al., 2004). Also, patients with IC/BPS have been found to have iNOS in the urothelium and inflammatory

infiltrates in the lamina propria which are suggested to be induced by inflammatory conditions. (Nussler and Billiar, 1993, Logadottir et al., 2013). The removal of the urothelium by ethanol may have reduced the levels of iNOS in these patients. However, capsaicin has been found to promote NO release by activating the TRPV1 channels on afferent nerves (Birder et al., 1998). The consequences of NO alterations by ethanol and capsaicin is unknown however bladder contractility in these patients may be enhanced. The reduction in detrusor contractile amplitude to carbachol may reflect the non-specific effects of capsaicin in high doses which can reportedly last for up to 15-hours (Maggi et al., 1989b, Cheng et al., 1993). Enhanced sensations of burning would be intensified rivaling the burning sensations produced by ethanol. In human skin, the heat pain threshold of polymodal C-fibres was reduced from 43.6°C to 36.9°C after treatment with capsaicin (Konietzny and Hensel, 1983). Regardless of these side effects that are reported to be temporary, based on available evidence, the long-term benefits can persist for days to weeks (Maggi et al., 1989a, Cruz et al., 1997, Soontrapa et al., 2003) . Overall, capsaicin seems have long-term favourable benefits for IC/BPS, the findings of this study would indicate that the reason appears to be due to the vehicle's intrusive actions on the bladder tissue which allow it to reach its target. Unfortunately, the side effects make it intolerable for these patients suggesting that an alternative and competent delivery system is required.

Summary

To summarise, RTX has not damaged the urothelial layer any more than its ethanol vehicle as there were no further changes to the release of mediators or LDH activity from the urothelial surface during treatment. There were also no further alterations to tissue integrity or the responses to purinergic, non-receptor mediated, muscarinic, nerve-mediated stimulation as a result of pre-treatment. The only exception was isolated strips of intact tissue that had a depressed response to purinergic stimulation following RTX pre-treatment which was thought to be due to the diffusion of NO from the urothelium/lamina propria to the detrusor. On the other hand, during treatment with capsaicin, ACh release was increased in addition to the already enhanced ATP levels and LDH activity produced by the ethanol vehicle (30%). Following pre-treatment with capsaicin, the urothelium/lamina propria also had enhanced ACh release under basal and stretched conditions while ATP release under these conditions remained similar to the ethanol vehicle. No further alterations were found in tissues responses compared to the ethanol vehicle for purinergic, non-receptor mediated or nerve-mediated

stimulation. However, the isolated urothelium/lamina propria had an enhanced contractile response to muscarinic stimulation while the response of the detrusor was depressed.

In conclusion, RTX has produced minimal changes to the bladder during and after treatment. There is considerable doubt as to whether it had been able to reach its sub-urothelial target or whether 10% ethanol is a suitable vehicle for this treatment. Other factors to be considered are its slower diffusion rate and onset, its concentration (50 nM), which may not be adequate and its ability to bind less effectively to pig and human tissue compared to rodents. Conversely, capsaicin has readily diffused to its sub-urothelial target. Although the majority of the side effects are produced by the ethanol vehicle, these are likely to be slightly enhanced by capsaicin. This presents tolerability concerns for IC/BPS patients. However, once the symptoms subside the long-term outlook is favourable.

Chapter 6:
The effects of resiniferatoxin on murine bladder function

6.1 Introduction

Suitability of the mouse bladder

Using pig and or human bladder for whole bladder studies is unpractical due to the lack of available whole human bladders and the size and bulk of using whole pig bladders. As a result, the majority of these studies use bladder strips that are limited to investigating contractile activity on a single plane and do not address the complexities of the volume and pressure relationship of the intact organ that occurs in a clinical setting (Damaser, 1999). To address this issue, rodents including rats, mice and guinea pigs are commonly used (Andersson et al., 2011). It has been proposed that the mouse bladder may be morphologically more similar to the human bladder due to the presence of intramural ganglia, which is lacking in the rat. However, unlike healthy human bladders, neurally released adenosine 5'-triphosphate (ATP) is a significant contributor to parasympathetic transmission. (Fabiyi and Brading, 2006, Andersson et al., 2011). C57BL/6 is considered to be one of the oldest and most extensively used mouse breeds for research (Bryant et al., 2008).

Nervous supply

In general, the nerve connectivity and supply of the urinary bladder is similar across all species (Keast et al., 2015). Pelvic ganglia (containing pelvic and hypogastric nerves) provides the majority of parasympathetic (cholinergic) and sympathetic (noradrenergic) innervation to the bladder receiving synaptic input from preganglionic neurons that are located in lumbar and sacral regions of the spinal cord (Keast, 1995). In mouse strains including C57BL/6, most of the cholinergic and noradrenergic output travels through the hypogastric nerve with a considerably smaller proportion traveling via the pelvic nerve. Similar to the human bladder, noradrenergic innervation of the mouse strain C57BL/6 has a sparse supply in the trigone, is rarely found in the detrusor and is absent from the urothelium, although pronounced innervation of the blood vessels supplying the bladder has been observed. Cholinergic innervation was found to be prevalent in the detrusor and urothelium (Wanigasekara et al., 2003).

Parasympathetic division

Like human bladders, parasympathetic outflow in the mouse is considered to provide the major excitatory output to the urinary bladder. Parasympathetic nerves release acetylcholine (ACh), ATP and nitric oxide (NO) to concomitantly contract the detrusor muscle while relaxing the urethra smooth muscle (Fujiwara et al., 2000, Fabiyi and Brading, 2006).

Pharmacological studies have revealed that contraction to neurally released ACh is predominantly facilitated by the M3 receptors while the M2 receptor has been shown to act indirectly on contraction by reversing sympathetically mediated relaxation (Choppin and Eglen, 2001, Choppin, 2002, Ehlert et al., 2005, Ehlert et al., 2007). In the rat bladder and human detrusor cells, carbachol-mediated stimulation of the M3 receptors results in increases to inositol triphosphate (Harriss et al., 1995, Kories et al., 2003). Comparable to the human detrusor, the contractile activity of the mouse detrusor in response to carbachol relies on both intracellular and extracellular calcium (Ca^{2+}) sources. However, it appears to be more reliant than human tissue on extracellular sources of Ca^{2+} via the L-type channel as nifedipine (L-type Ca^{2+} channel blocker) reduced contractile response to carbachol by 73% compared to a 25% reduction in the human detrusor (Wuest et al., 2007).

Unlike the healthy human bladder in which nerve-mediated contraction is exclusively cholinergically mediated, nerve-mediated contraction in the mouse bladder employs both a purinergic and cholinergic component (Sibley, 1984, Fabiyi and Brading, 2006, Ehlert et al., 2007). The purinergic component has been reported to contribute to approximately 38% of the nerve-mediated response when whole mouse bladders were stimulated with electrical field stimulation (EFS). (Ehlert et al., 2007). In P2X1 knock out mice, atropine was able to almost abolish nerve evoked force in strips of mouse smooth muscle demonstrating that this receptor alongside M3 is also responsible for contraction in this tissue (Choppin and Eglen, 2001, Heppner et al., 2009). Heppner et al. (2009) has further suggested that neurally released ATP mediates a rapid rise in contractile force whereas muscarinic receptor activation by ACh determines the duration of force. The sizeable purinergic component (compared to the healthy human bladder) of the mouse bladder may be useful to study bladder pathology where an increase in neurally released ATP plays a more prominent role in the human bladder (Palea et al., 1993, Bayliss et al., 1999).

The mouse bladder urethra relaxes to electrical field stimulation in which relaxation was

abolished entirely in the presence of nitric oxide synthase (NOS) inhibitor L-NOARG suggesting that NO exclusively relaxes the bladder outlet. This was accompanied by a dominant increase in 3,5 cyclic guanine monophosphate (cGMP) immunoreactivity in urethral tissues stimulated by NO donors (Fujiwara et al., 2000).

Sympathetic division

The role of the sympathetic nervous system is to allow the bladder to relax while increasing bladder outlet resistance during the storage phase of the micturition cycle (Yoshimura and de Groat, 1997). It is generally accepted that adrenergic relaxation of the human bladder is mediated by the β3-adrenoceptor, while in the mouse, sympathetic mediated relaxation may occur via β2 and β3-adrenoceptors (Deba et al., 2009, Wuest et al., 2009, Palea et al., 2012). Functional α-adrenoceptors have also been found in the mouse bladder that contributes to its storage function (Chen et al., 2005a).

Afferent division

The afferent division reports sensory information from the bladder regarding distension, contraction and its contents back to the CNS (Wyndaele, 2010). Multiple subtypes of afferent nerves have been identified in the mouse bladder (C57BL/6) by immunohistochemistry including those that resemble Aδ and C-fibres. The distribution of afferent fibres in the mouse bladder is comparable to the human bladder with Aδ -fibres primarily located in the detrusor with only a few of these fibres running in the sub-urothelium. Nerve fibre staining for CGRP, identified as C-fibres, were found more densely in the sub-urothelium at the bladder base, throughout the basal urothelial layer extending to below the umbrella cells (Rahnama'i et al., 2017).

The urothelium

The mouse urothelium contains approximately three layers compared to human which is approximately 5-7 layers (Phillips and Davies, 1980, Treuting et al., 2012). The mouse urothelium contains basal, intermediate and umbrella cells, including uroplakins as well as distinct glycosaminoglycan (GAG) layer (Ramesh et al., 2004, Zarghooni et al., 2007, Aboushwareb et al., 2009). Cultured mouse urothelial cells release ATP, while whole bladder distension results in the luminal release of ATP, ACh and NO, moreover, detectable levels of

ATP and ACh can be obtained from the serosal surface of the bladder (Birder et al., 2002, Daly et al., 2014, West et al., 2018). The physiology of the urothelium found in mouse bladders could be useful for determining treatment outcomes in bladder pathology that is characterized by a thinner urothelium (Slobodov et al., 2004, Tomaszewski et al., 2001).

The mouse urothelium is known to express all five muscarinic subtypes and multiple nicotinic receptors with immunolabelling identification of the M2 receptor restricted to the umbrella cells (Zarghooni et al., 2007). Immunohistochemistry has also identified P2X3 receptors on afferent nerve fibres that project in between the urothelial cells in mice which may be the recipients of luminal ACh and ATP (Vlaskovska et al., 2001).

Thus, the available evidence suggests that the mouse bladder is a useful tool that can provide some insights into whole bladder functioning although there are some differences in physiology and signalling and when compared with the human bladder.

The mouse bladder and RTX

As mentioned in the previous chapter, much of the animal research on the effects of RTX or capsaicin has been obtained in the rat and information regarding the intravesical use of RTX in mouse bladders let alone its common vehicle (10% ethanol) is scarce (Appendino and Szallasi, 1997, Andersson et al., 2011, Uvin et al., 2012). Nonetheless, RTX binds to its receptor with similar binding affinities in the bladder of both mouse and rat (Szallasi et al., 1993).

In the mouse bladder, the instillation of 1 nM RTX for 30-minutes resulted in decreases in spinal *c-fos* expression 24-hours after treatment (Janssen et al., 2016). In another study, it was found that 1μM RTX delivered intravesically (30 mins) significantly increased the bladder sensory threshold in C-fibres but not in Aδ- fibres 24-hours after its application (Bicer et al., 2014). In other experiments not using intravesical delivery, the presence of RTX on mouse isolated bladder strips and cultured mouse urothelial cells evoked NO release (Birder et al., 2001). It was also found in mouse DRG neurons, that RTX increased CGRP immunoreactivity that was concentration dependent (0.3-30 nM), without increasing the number of CGRP positive cells 24-hours after exposure (Jakab et al., 1994).

Transient receptor potential vanilloid 1 (TRPV1) channels have been found in close association to the afferent fibres in the mouse bladder (Vlaskovska et al., 2001). As RTX has been found

to reduce its binding sites in the bladder after 24-hours, TRPV1 knockout mice may provide some insights as to what may occur 24-hours after treatment (Goso et al., 1993). In TRPV1 knock out mice, spinal *c-fos* expression was not changed in inflammatory conditions compared to TRPV1 +/+ mice indicating that this receptor has a crucial role in pain and subsequent overactivity associated with cystitis (Charrua et al., 2007). This is in addition to the findings of Birder et al. (2002) that revealed TRPV1 knockout mice have increased bladder capacity, inefficient voiding, reduced spinal cord *c-fos* in response to bladder filling and insensitivity to capsaicin (under anaesthesia) compared to wild-type mice for this study. Mice devoid of TRPV1 channels have also been found to have attenuated afferent activity in low threshold afferents in response to bladder filling (Daly et al., 2007).

Other indications of what may occur in the mouse bladder treated with RTX is systemic treatment with capsaicin. In newborn mice, there were significant reductions found in Aδ fibres and unmyelinated fibres, presumably C-fibres, three to five months following subcutaneous injections of capsaicin (50 mg/kg in 50 µl) (Hiura and Sakamoto, 1987). Maggi et al. (1987b) also identified that systematic capsaicin (55 mg/kg) significantly reduced the SP content in the mouse urinary bladder four days after treatment.

From the evidence that is available, the effects of RTX or capsaicin on mouse tissues does not seem to differ drastically from the rat tissue (see the introduction in the preceding chapter). Furthermore, the thinner urothelium and the more dominant purinergic signalling is a similar feature that is found in interstitial cystitis/bladder pain syndrome IC/BPS which makes it an ideal model to assess intravesical treatment for this study (Phillips and Davies, 1980, Palea et al., 1993, Slobodov et al., 2004, Ehlert et al., 2007).

6.2 Objectives

The objectives of this study were to investigate the effects of intravesical RTX (50 nM) and 10% ethanol (vehicle control) in the mouse bladder *in vivo* and to examine whole bladder function *in vitro*. Specific aims were;

1. To investigate voiding patterns immediately before and 24-hours after intravesical treatment with 0.9% saline (control), 10% ethanol (vehicle control) or 50 nM RTX.
2. To quantify ATP and ACh release from luminal and serosal isolated bladders 24-hours after treatment with saline, the vehicle or 50 nM RTX.
3. To examine nerve-induced bladder contractions 24-hours after treatment and assess the contributions made by the purinergic, muscarinic, and nitrergic systems.
4. To identify any changes that may have occurred to bladder compliance, spontaneous activity and contraction/relaxation mechanisms using pharmacological agents 24-hours after the various intravesical treatments.

6.3 Materials and methods

Ethical approval

Approval for this project was obtained from the University of Queensland Animal Research Ethics Committee (Approval # Bond/150/17)

Animals

Eight-week-old female C57BL/6J mice were acquired from the Animal Resource Centre (Canning Vale, WA, Australia) and were housed at Bond University Animal House (Bond University, Qld, Australia).

C57BL/6J is a widely used strain in research and these mice were housed for one week prior to any experimental procedures in a controlled environment with free access to food and water, held at a constant temperature of 23 °C with 12-hour light/dark cycles. Mice were then allocated into three treatment groups consisting of a control, vehicle control or RTX group, **(Figure 6.1)**.

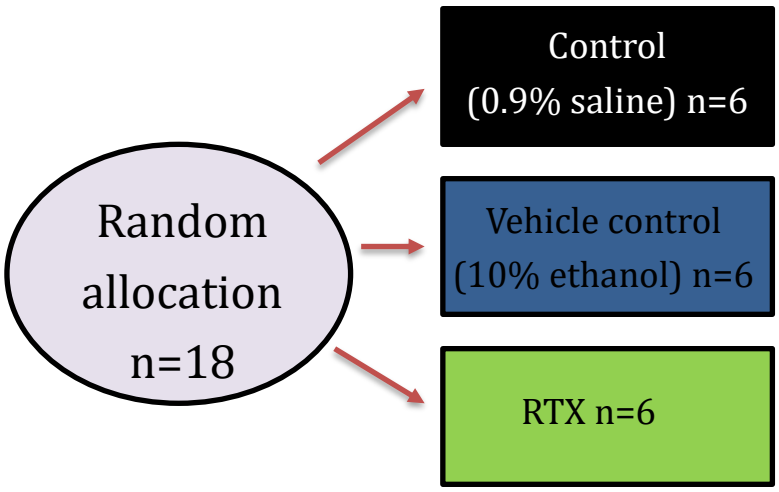

Figure 6.1: The random allocation of mice into three treatment groups.

Voiding pattern analysis

The commencement of this study involved examining the voiding behaviours of all mice immediately before and 24-hours after intravesical treatment using Filtech' hardened ashless filter paper, Quantitative 2 um grade 225 (Interpath Services, Victoria, Australia). At the change-over of the light/dark cycle (7:30 am), a single mouse was placed in a filter paper lined cage with free access to food and water for 4-hours immediately before treatment and 24-hours after treatment. This protocol was based on previous studies regarding the voiding patterns of mice of the same breed (Yu et al., 2014, Bjorling et al., 2015, West et al., 2018). After 4-hours, the filter paper was collected and urine spots were detected using Molecular Imager Chemidoc XRS ultraviolet transilluminator (#720BR1293 BioRad, California USA). Based on the protocol by Yu et al. (2014), the captured voiding images were then thresholded and their area was calculated by using the "analyze particles" function in Image J software (http://imagej.nih.gov.ij). The results of the analysis by Image J could be used to assess the total volume voided, the volume of the largest area of urine termed the "primary void area", the voiding frequency and the number of small spots <1 µL **(Figure 6.2A)**. To determine the volume of urine (µL), known quantities of mouse urine (1-100 µL) were applied to the filter paper and a linear relationship was found between volume and area (r^2 = 0.99) **(Figure 6.2B&C)**. Voiding frequency was determined by counting the urine spots whose area was larger than 0.005 m^2 which was the area of the smallest spots that were still visible macroscopically.

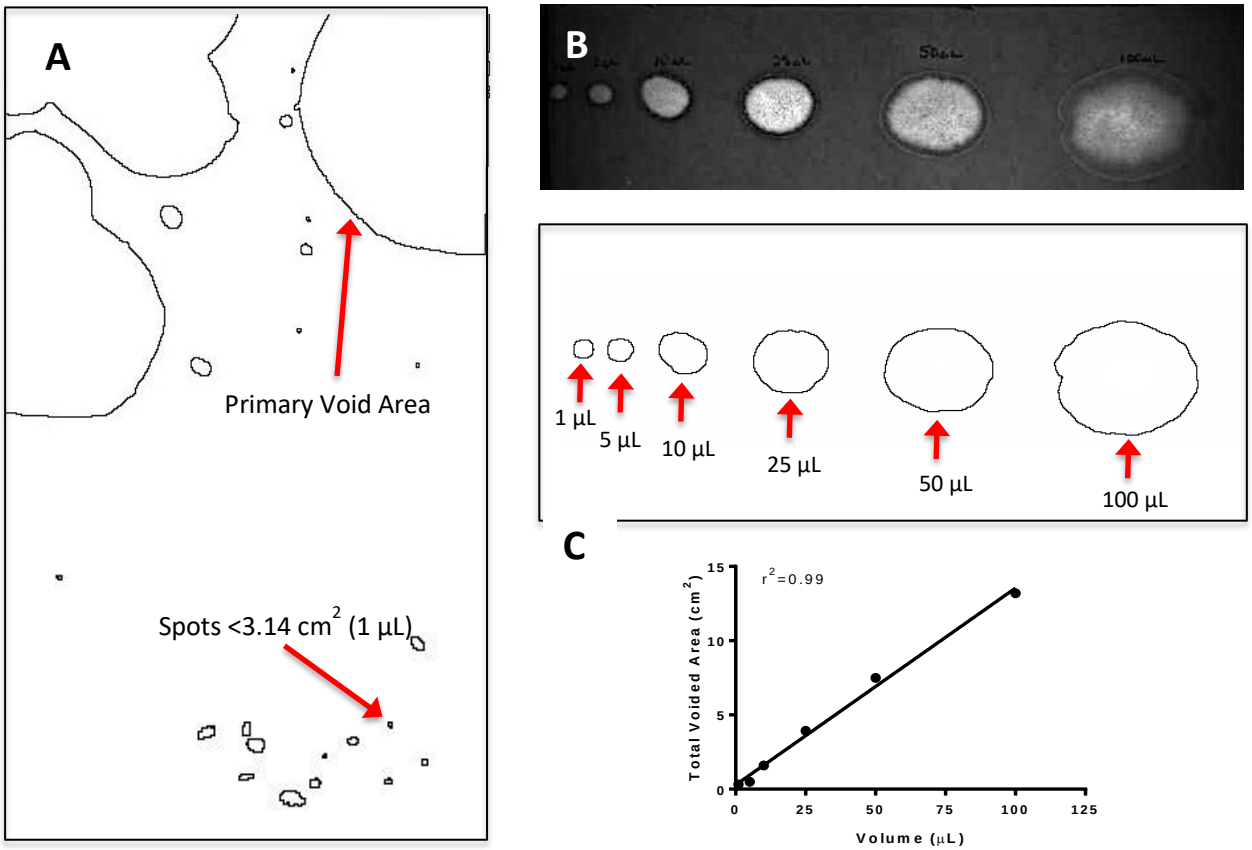

Figure 6.2: Illustration of the results produced by Image J "analyze particles" function (A), the captured image under UV light of known quantities of mouse urine and the Image J results (B) and the linear relationship between volume and area (C) that was used to calculate the volume of urine.

Intravesical treatment

Immediately following the first voiding pattern analysis, a 0.9% saline solution (control), a 10% ethanol solution (vehicle control) or 50 nM RTX (dissolved in 10% ethanol) was instilled into the mouse bladder for 30-minutes reproducing clinical treatment. Before the instillation of treatment, the mouse was anaesthetized with 1-3% isoflurane gas. Inhalation of isoflurane gas is commonly used as an anaesthetic because of its simplicity to administer, low complication rate, its rapid onset/recovery and its ease of depth control (Szczesny et al., 2004). Isoflurane inhalation had also been shown to suppress the micturition reflex which additionally assists with intravesical delivery of drugs (Matsuura and Downie, 2000).

Once the mouse was anaesthetized and moments prior to intravesical delivery of treatment, the mouse bladder was gently massaged to expel any remaining urine and to lubricate the urethra.

Bladder catheterization was completed using a sterile intravenous 22x1 gauge catheter (Terumo, Japan) that was inserted into the urethra based on the technique that was reported by Reis et al. (2011).

Briefly, the mouse was placed in dorsal recumbency and the catheter tip was carefully introduced into the external urethral orifice by 3 mm, parallel to the distal urethra in a cranial to caudal direction **(Figure 6.3a)**. To gain entry to the urethra the catheter was then aligned by a 180 ° circular movement of the proximal catheter while keeping the distal portion static **(Figure 6.3b)**. Once the catheter was aligned, it was introduced 7 mm into the bladder **(Figure 6.3c)**. Preparations of 0.9% saline, 10% ethanol and 50 nM RTX were instilled into the bladder at a volume of 30 µL before catheter removal.

After this process was complete, the mouse was placed in a recovery cage that lined with filter paper and food, but no water for 30-minutes. The absence of water allowed the instillate to remain undiluted and in the bladder for the pre-determined period. After 30-minutes had elapsed, micturition was induced to expel the treatment fluid from the bladder and the mouse was housed with free access to food and water for a further 24-hours. During this time the mouse was monitored post-treatment for signs of distress or pain. The following morning, the same mouse repeated another voiding pattern analysis at 7:30 am for 4-hours and was then humanely sacrificed by cervical dislocation in accordance with the ethical committee approval and the guidelines of the National Health and Medical Research Council.

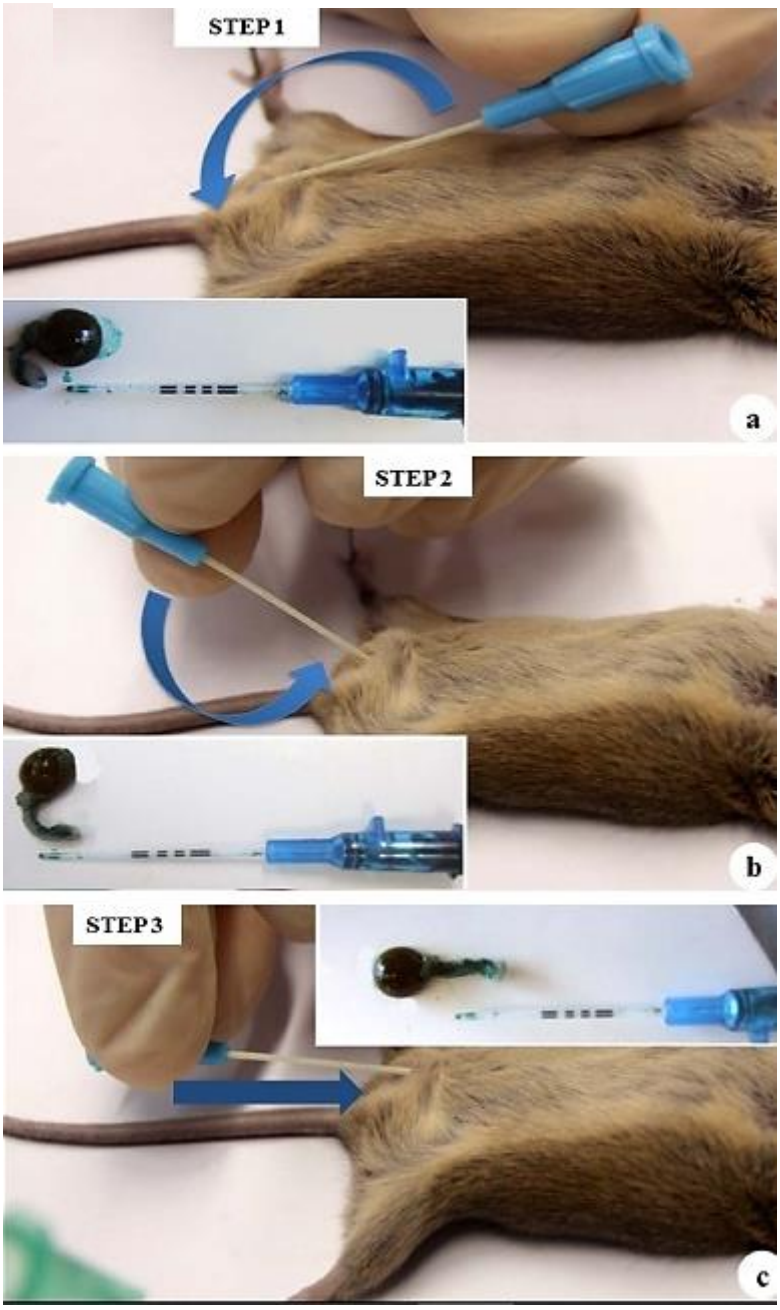

Figure 6.3: Stepwise instructions for catheterization of the mouse bladder. a-placement of the catheter tip 3 mm into the external urethral orifice. b-a 180 ° circular movement aligns the catheter with the urethra for entry and c- introduction of the catheter into the urethra. Inset pictures from a,b & c demonstrate the position of the bladder with regards to the catheter for each step. The blue arrows show the movement of the catheter for each step (reproduced with permission from the publisher, (Reis et al., 2011)

Whole bladder isolation

After cervical dislocation, an incision was made in the upper abdomen and the skin and peritoneum was removed revealing the gastrointestinal tract. This was carefully removed exposing the kidneys and organs of the lower abdomen. The vertebra was severed at approximately L 1-2 and the legs and tail were removed. The portion containing the kidneys and the bladder was placed in a dissection dish filled with Krebs-bicarbonate solution at 37 °C that was gassed with 95% O_2 and 5% CO_2.

A dissection microscope (WPI, PZMIII) was used to assist in the removal of the oviduct, uterine horn, uterine fundus. The surrounding connective tissue around the bladder was also removed revealing the pubic symphysis. The pubic symphysis was severed and the pelvis and remaining hind legs were removed either side of the urethra. With the bladder now exposed, the ureters were ligated proximal to the bladder with silk suture and a three-way catheter was introduced into the urethra and into the bladder that was also secured by silk sutures **(Figure 6.4B)**. The urethra was then dissected away from the remaining vaginal tissue and uterus, the ureters were cut above their ligation sutures and the isolated bladder was removed from the pelvic area and placed in a 10 mL organ bath containing Krebs-bicarbonate solution at 37 °C that was gassed with 95% O_2 and 5% CO_2.

The three-way catheter that was inserted into the bladder had one tube that was attached to an infusion pump to allow filling of the bladder. The second tube was attached to a pressure transducer that recorded intravesical pressure (mmHg) and a third tube was used for outflow that had a tap which allowed for bladder emptying **(Figure 6.4A)**. Changes to intravesical pressure were detected by the pressure transducer (Global Towns, USA) and recorded by a Powerlab recording system (ADinstruments Ltd.) via an Octal Bridge Amp (ADinstruments Ltd.) and analysed using LabChart (version 7.0.3) software (ADinstruments Ltd.)

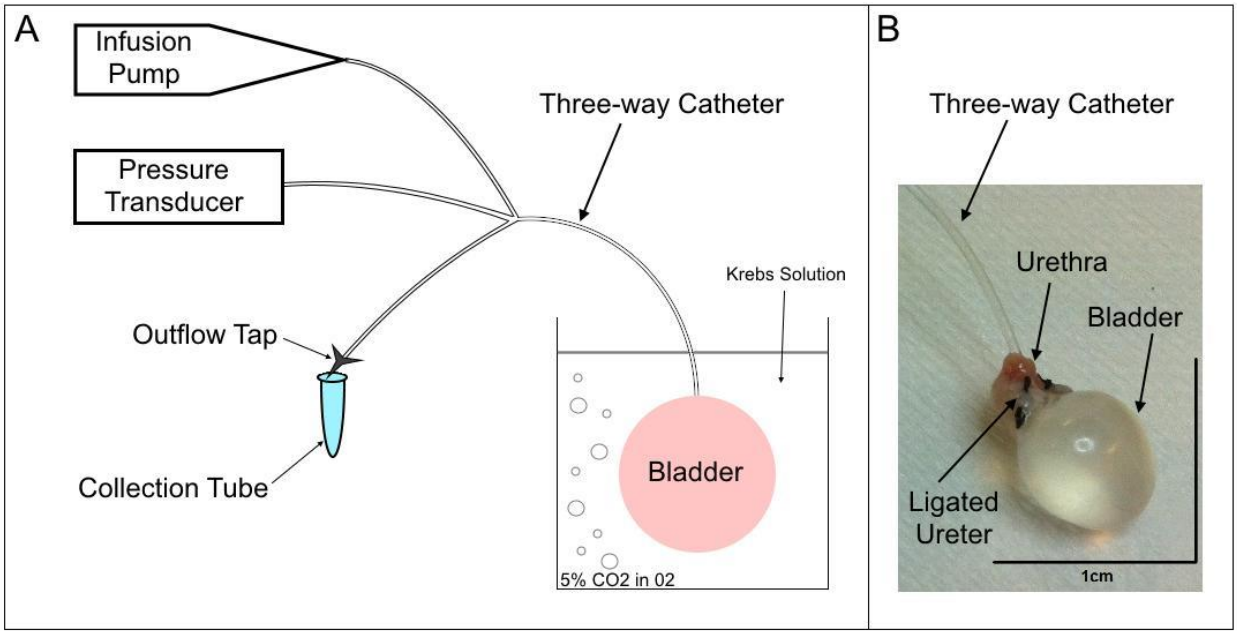

Figure 6.4: A- Illustrates the isolated bladder connected to a 3-way catheter in which one tube connects to an infusion pump, another to a pressure transducer while the last tube allows for ouflow of the bladder contents. B- Is an image of the isolated mouse bladder that is connected to a 3-way catheter via the urethra.

The isolated bladder was then distended to 40 mmHg using saline solution infused at 30 µL/min to check for any potential leaks and viability. Once 40 mmHg was attained, the outflow tap was opened which allowed for passive emptying of the bladder contents. The bladder was then distended to 20 mmHg and allowed to empty passively twice more before a final distention to 20 mmHg in which the bladder was then allowed to stabilize for approximately 1-hour. After this hour, the baseline values of the closed system (≤5 mmHg) were subtracted from the pressures during distension and pharmacological intervention to record the changes occurring to intraluminal pressure.

Bladder compliance and spontaneous activity

The effect of intravesical treatment on bladder muscle compliance was calculated by plotting bladder volume vs. bladder pressure. In addition, spontaneous activity of the whole bladder was recorded after the bladder had stabilized for 1-hour. Amplitude was recorded from peak to trough of a contraction and frequency was recorded as the number of contractions per minute that exceeded 30% of the peak amplitude which is a protocol adaptation from Imai et al. (2001).

Mediator release

Following the initial bladder distension to 40 mmHg to confirm viability, the organ bath was washed and replaced with 8 mL of fresh Krebs-bicarbonate solution and the bladder was distended to 15 mmHg (20 mmHg - baseline <5 mm Hg) which reportedly represents the micturition threshold of 15 mmHg in mice (Daly et al., 2007). Once the desired pressure was achieved, the bladder was allowed to empty passively and the contents were collected to analyse luminal mediator release 24-hours after intravesical treatment. A sample of the Krebs solution surrounding the bladder was also taken to compare mediators from the serosal side of the bladder. Both luminal and serosal samples were placed in a microfuge tube on ice and were stored at -20°C for later analysis. After baseline pressures returned to initial values (10-minutes), the organ bath was washed and replaced with fresh Krebs-bicarbonate solution and this process was repeated. The collected samples were analysed for the presence of ATP and ACh using the commercially available kits that have already been described in chapter two.

Electrical field stimulation

Bladder contraction mediated by the efferent nerves was investigated by EFS 24-hours post-treatment. After the bladder had been distended to 20 mmHg for the final time and had stabilized for approximately 1-hour, platinum electrodes were placed either side of the bladder in the organ bath **(Figure 6.5)**. Nerve-mediated stimulation (50V, 0.1 ms delay, 0.2 ms pulse duration) was delivered as a 5-second train every 100-seconds at 1,5,10 and 20Hz. For every frequency, four contractile spikes were produced and amplitude was measured as the change in pressure from baseline.

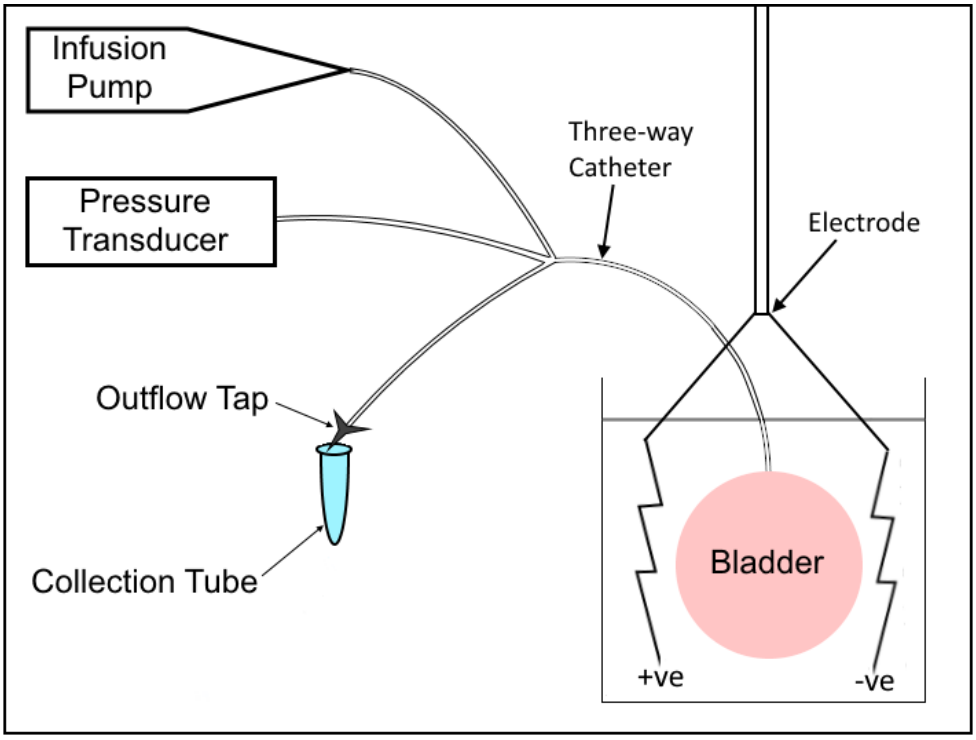

Figure 6.5: Demonstrates the set up for EFS. While the bladder is still connected to the 3-way catheter, platinum electrodes are placed either side of the bladder to record the responses to nerve-mediated stimulation.

As ATP, ACh and NO play a part in mouse bladder contraction, their contribution to nerve-mediated contraction was also assessed (Daly et al., 2014). EFS was repeated at 20 Hz in the presence of the nitric oxide synthase inhibitor L-NNA (10 µM). Following on from L-NNA, the muscarinic antagonist atropine (1 µM) was added to the organ bath and EFS at 20 Hz was repeated. Lastly, α,β m-ATP (10 µM) to desensitize the PX3 receptors with was additionally added to the bath and EFS was again repeated at 20 Hz. It is important to note that throughout the EFS process, the bladder was not washed in between the application of pharmacological agents.

Response to pharmacological intervention

After the initial frequency responses to EFS, the bladder was washed several times and allowed to stabilize for approximately 1-hour. To assess the influence that intravesical treatment had on contraction/relaxation mechanisms, the contractile responses of mouse bladders were assessed after the addition of ATP (1 mM) to observe the peak responses to purinergic stimulation and were washed to baseline pressures.

After the tissues had stabilized, the muscarinic receptors were stimulated with cumulative-concentrations of carbachol which ranged from 10 nM to 10 µM and the contractile response was measured. Following carbachol stimulation and a subsequent washout period, the bladder returned to its pre-carbachol baseline pressures. The bladder was then re-distended to 15 mmHg in preparation for cumulative concentrations of isoprenaline (β-adrenoceptor agonist) to measure the relaxation response. Passive tension was chosen as the stimulus for examining the reduction in bladder tension in response to isoprenaline as it was reported by Michel and Sand (2009) that isoprenaline was significantly less potent and less effective against carbachol stimulation in the rat bladder. The cumulative concentrations for isoprenaline ranged from 500 pM – 5 µM.

After all experiments had been carried out, the tissue was washed to baseline pressures and KCl (60 mM) was added to the organ bath to measure non-receptor mediated contraction. After the peak response to KCl had been recorded the experiments on the isolated mouse bladder concluded.

Statistical analysis

For these studies, data was expressed as mean ± standard error of the mean (SEM) and was analysed by one-tailed T-Tests or a one-way ANOVA with either Bonferoni, Tukey-Kramer or Dunnet compare all vs control multiple comparisons post-hoc test using Graphpad Instat (version 3.10) (Graph Pad software, San Deigo, USA). Curve analysis was performed with multiple comparisons F-test using GraphPad Prism (version 7.03) (Graph Pad software, San Deigo, USA). Significance was defined as *P<0.05, **P<0.01, ***P<0.001. Specific statistical tests are indicated in each figure legend.

6.4 Results

Voiding pattern analysis

Voiding pattern analysis evaluated the voiding behaviours of mice over a 4-hour period before and after (24-hours) intravesical treatment with saline (control), 10% ethanol (vehicle control) and RTX (50 nM). The parameters for the analysis were calculated by photographic capture of the used filter paper under UV light shown in **Figure 6.6A**. The calculated total volume of urine, the volume of the primary void, the total number of voiding events and the number of small urine spots <1 µL over a 4-hour period are also shown **(Figure 6.6B)**.

Representative images for each experimental group show the voiding patterns that occur over a 4-hour period immediately before and 24-hours after pre-treatment with saline **(Figure 6.7A&B)**, the ethanol vehicle **(Figure 6.8A&B)** and RTX **(Figure 6.9A&B)**.

For saline **(Figure 6.7C-F)** and vehicle control **(Figure 6.8C-F)** treated mice there were no changes to the total volume of urine, the volume of the primary void, the total number of voiding events and the number of small urine spots <1 µL before and 24-hours after treatment.

However, pre-treatment with RTX significantly increased the voiding frequency by approximately 100% **(Figure 6.9E),** while the volume of the primary void area was significantly reduced by 30% **(Figure 6.9D)**. This corresponded to an increase in the number of small spots <1 µL (pre-Tx 10±2.9 vs. post-Tx 20.7±4, n=6, P<0.056) with no change to the total volume voided before and 24-hours after treatment with RTX **(Figure 6.9F)**.

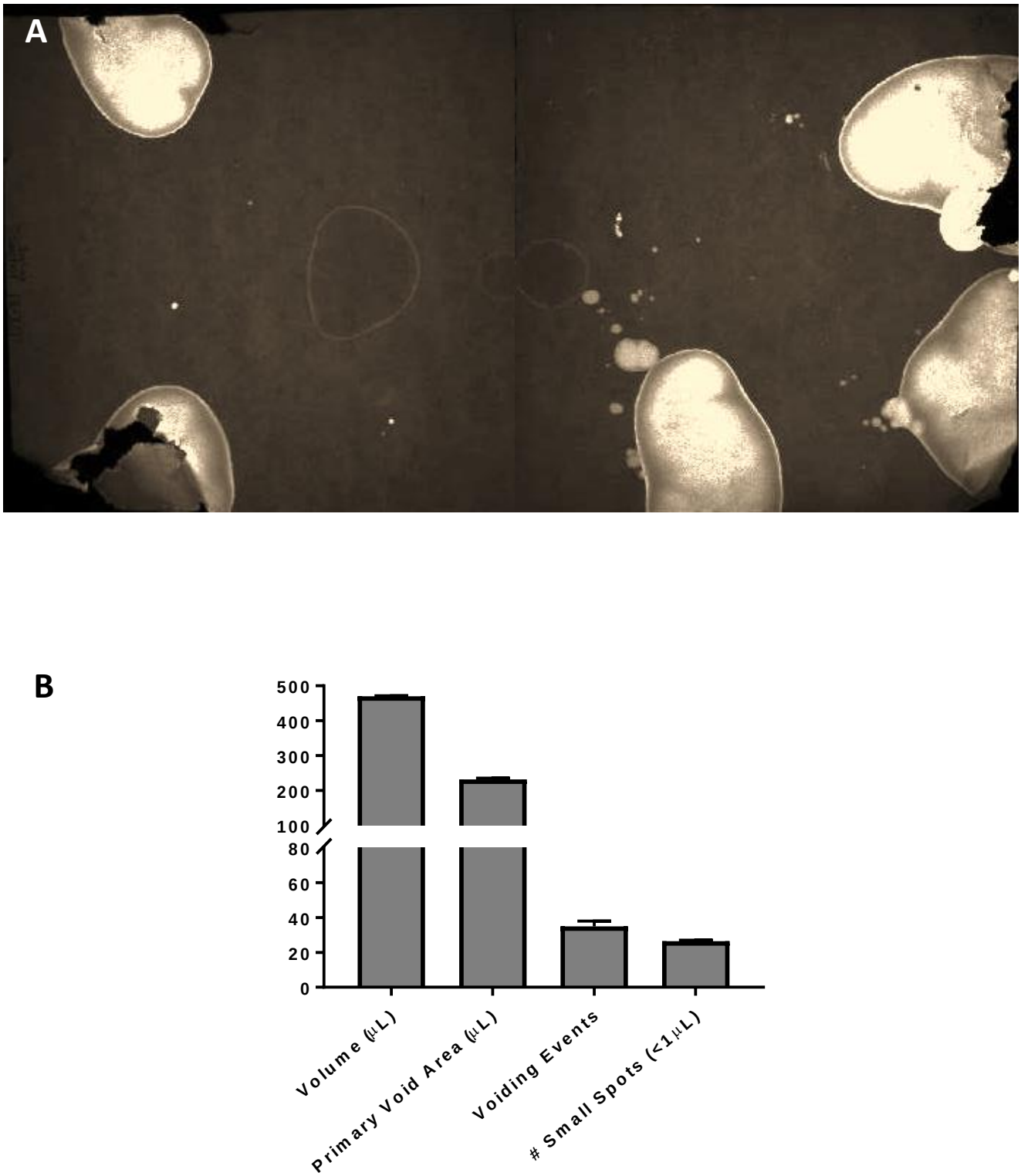

Figure 6.6: Depiction of a voiding pattern analysis where A- represents the photograph taken under UV light after a 4-hour period which highlights the voiding pattern. B-demonstrates the measurements that can be obtained from the photograph to produce a complete analysis.

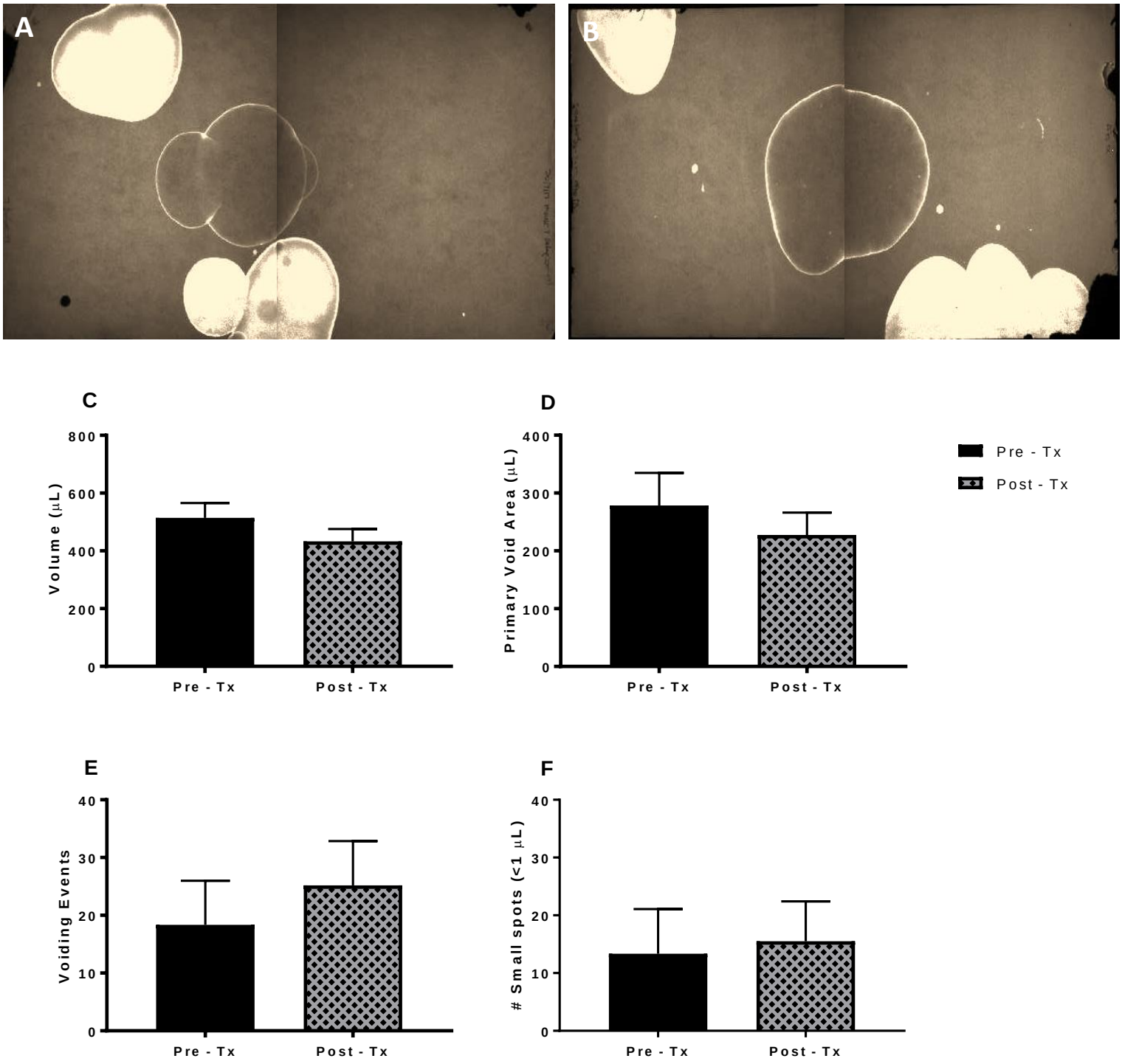

Figure 6.7: Represents the voiding pattern analysis before and 24-hours after intravesical treatment with 0.9% saline (control). A- represents a single photograph of filter paper taken under UV light after a 4-hour period immediately before treatment and B- 24-hours later. C, D, E, F- show the mean calculated values after analysis that include total volume voided (C), the volume of the primary void (D), the number of voiding events (E) and number of small spots <1 µL (F). Data is represented as mean±SEM and was analysed by a paired one-tailed t-test (n=6).

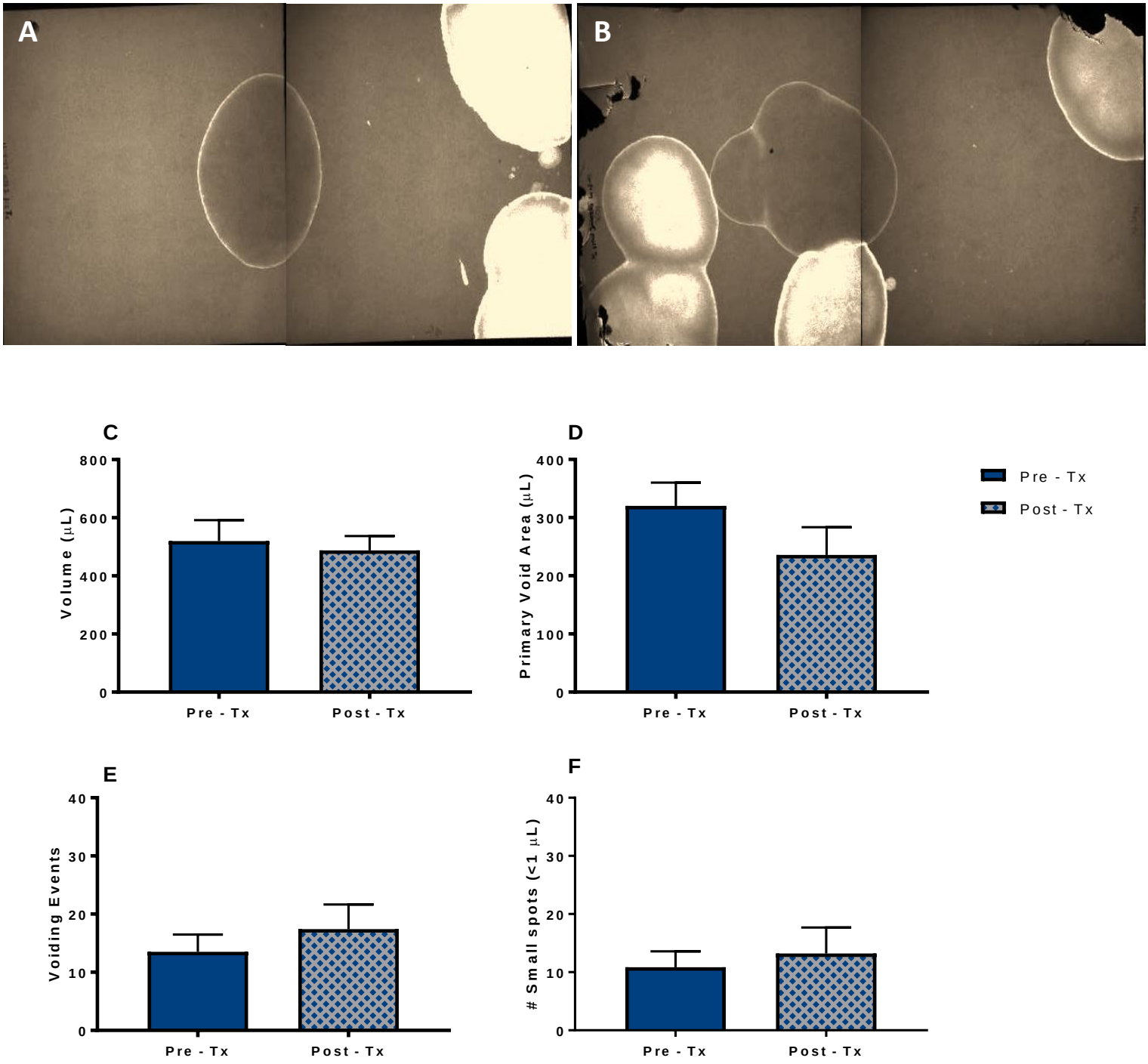

Figure 6.8: Represents the voiding pattern analysis before and 24-hours after intravesical treatment with 10% EtOH (vehicle control), A- represents a single photograph of filter paper taken under UV light after a 4-hour period immediately before treatment and B- 24-hours later. C, D, E, F- show the mean calculated values after analysis that include total volume voided (C), the volume of the primary void (D), the number of voiding events (E) and number of small spots <1 µL (F). Data is represented as mean±SEM and was analysed by a paired one-tailed t-test (n≥5).

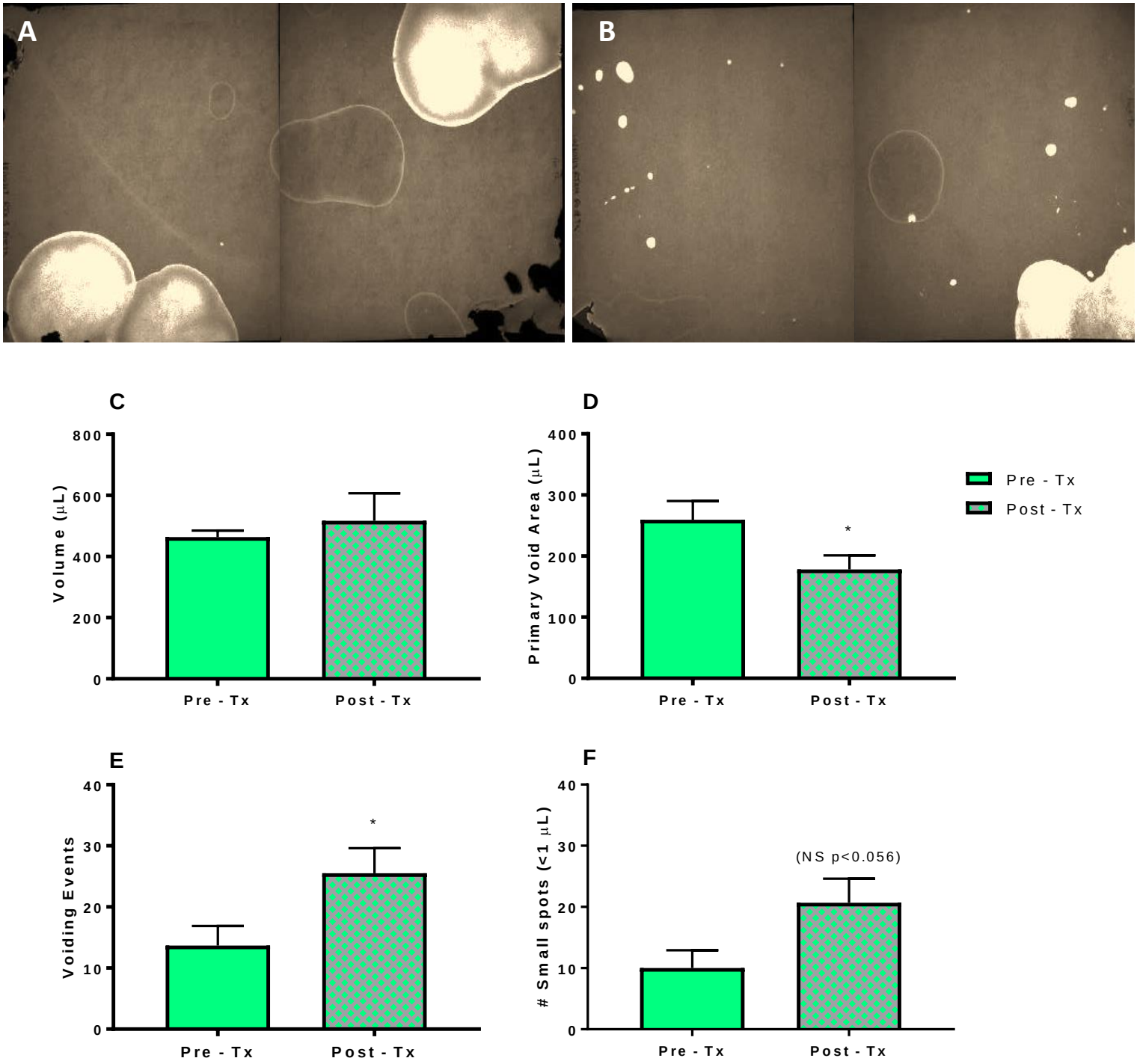

Figure 6.9: Represents the voiding pattern analysis before and 24-hours after intravesical treatment with 50 nM RTX, A- represents a single photograph of filter paper taken under UV light after a 4-hour period immediately before treatment and B- 24-hours later. C, D, E, F- show the mean calculated values after analysis that include total volume voided (C), the volume of the primary void (D), the number of voiding events (E) and number of small spots <1 µL (F). Data is represented as mean±SEM and was analysed by a paired one-tailed t-test (n=6), (*P<0.05 Pre-Tx vs. Post-Tx).

The general effects of treatment

The general effects of intravesical treatment were assessed in mice by evaluating any changes in body weight immediately before and 24-hours after intravesical treatment and by recording the volume of water consumed immediately after treatment for a period of 24-hours.

There were no changes to bodyweight between all mice in the saline, the vehicle control or RTX groups before and after treatment **(Figure 6.10A)**. Similarly, there were no changes to water consumption between all groups over the 24-hour period after treatment **(Figure 6.10B)**.

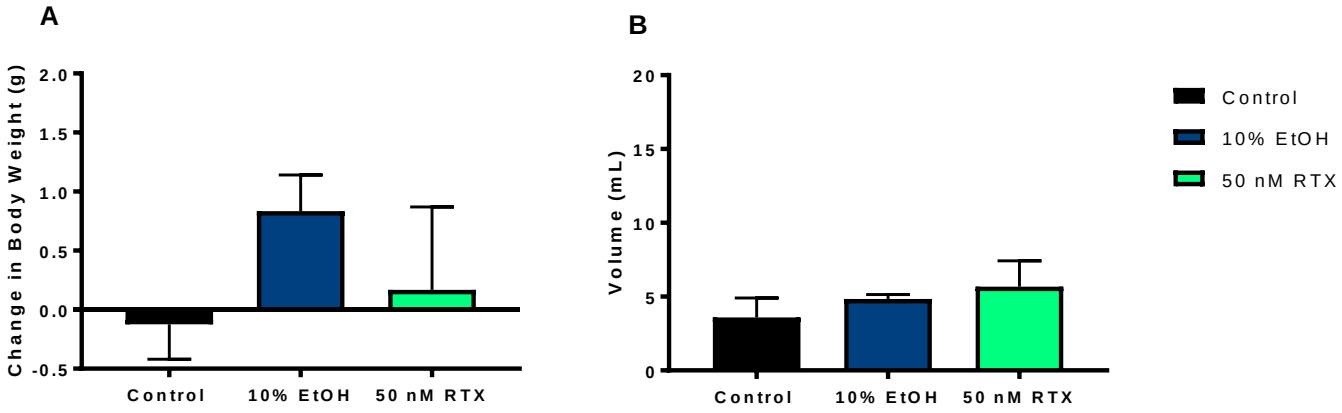

Figure 6.10: The recorded changes to bodyweight before and 24-hours after treatment (A) and water consumption (B) over the 24-hour period after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX. Data is represented as mean ± SEM analysed by a one-way ANOVA (n=6).

Bladder compliance

To assess if there were any changes in muscle compliance 24-hours after intravesical treatment, the isolated mouse bladders were distended to intraluminal pressures of 15 mmHg to create a pressure vs. volume curve. Overall, there were no differences in bladder capacity between mice that had been pre-treated with saline, the ethanol vehicle or RTX, although capacity in the vehicle control mice was lower and the value was almost statistically lower than the saline pre-treated mice (184.2±20.9 µL), the P value being (0.08). **(Figure 6.11)**.

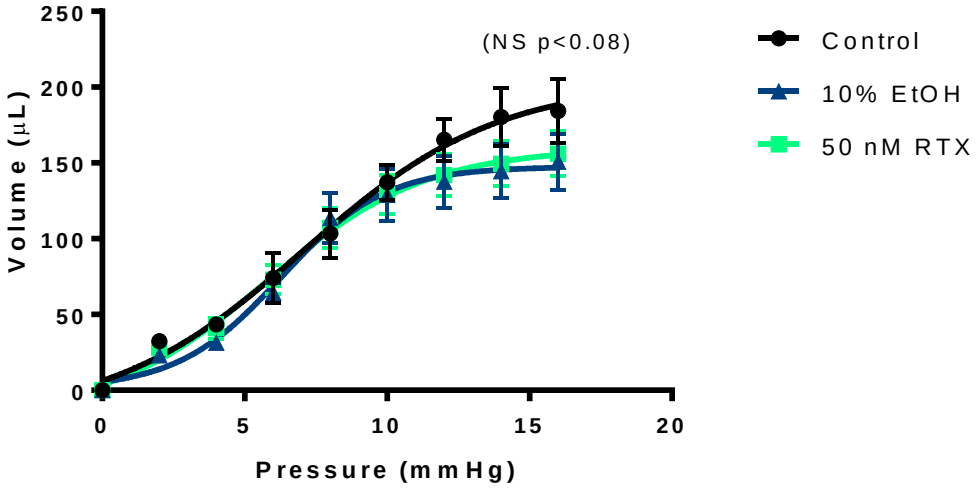

Figure 6.11: The volume-pressure curve after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX. Represented as mean ± SEM analysed by one-way ANOVA with Tukey multiple comparisons post-hoc test (n=6).

Spontaneous activity

After the initial equilibration period, spontaneous phasic contractions were observed in all bladders that had been intravesically treated 24-hours prior as shown in **Figure 6.12**. The amplitude of spontaneous contractile activity was significantly enhanced in bladders of mice that had been pre-treated with the ethanol vehicle when they were compared to saline pre-treated bladders (saline 0.23±0.09 vs 10% EtoH 2.03±2.01, n=6, P<0.05) **(Figure 6.13A)**. However, no statistical change in amplitude could be determined when the saline or ethanol vehicle pre-treated bladders were compared to bladders pre-treated with RTX. No alterations to the frequency of spontaneous events were observed in any of the experimental groups **(Figure 6.13B)**.

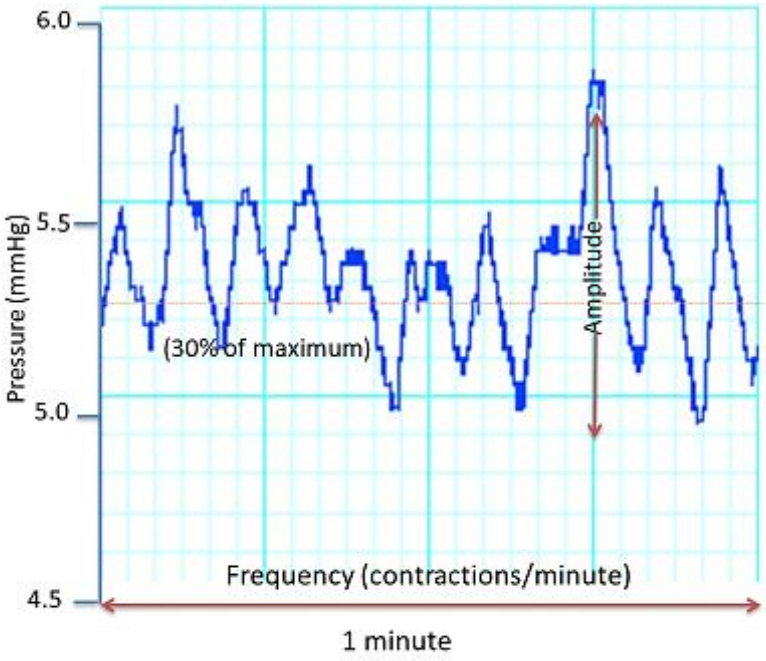

Figure 6.12: Illustration of the spontaneous activity that was observed in the isolated mouse bladder

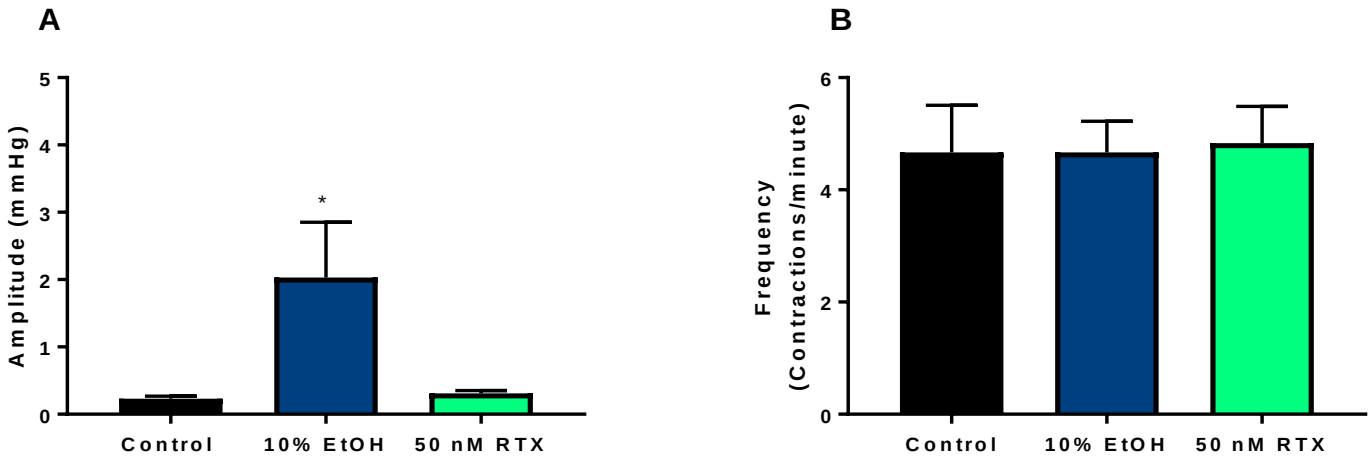

Figure 6.13: The (A) amplitude and (B) frequency of spontaneous contractions 24-hours after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX. Measurements were made after the bladder had stabilized for 1 hour. Values are represented as mean ± SEM analysed by one-way ANOVA with Tukey-Kramer multiple comparisons post-hoc test (n=6), (*P<0.05 saline vs 10% EtOH).

Mediator release

To observe any changes to distension induced ATP and ACh release that may have occurred 24-hours after intravesical treatment, samples of luminal and serosal fluid were collected at 15 mmHg for further analysis. Samples were collected from two consecutive distensions after recovery from the initial distension to 40 mmHg. The amounts of ATP and ACh released by both the luminal and serosal sides of the bladder were expressed as the total amount released by each side to account for the variance in volumes of the available luminal (<0.2 mL) and serosal (8 mL) sample fluid.

The total amount of luminal ATP release was significantly enhanced in the ethanol vehicle and RTX pre-treated bladders by 8-fold and 11-fold respectively when compared to the saline pre-treated bladders **(Figure 6.14A)**. Luminal ATP release was similar between bladders that had been pre-treated with the vehicle and RTX **(Figure 6.14A)**. While levels of ATP found in the serosal fluid appeared to be enhanced by the ethanol vehicle or RTX compared to saline pre-treatment, the increase was not statistically significant **(Figure 6.14B)**.

No variation to total luminal ACh release could be found between bladders in all experimental groups **(Figure 6.14C)**, however, a significant decrease in serosal ACh could be observed in bladders that had been pre-treated with RTX when compared to saline (saline 4±1.0 pM vs 50 nM RTX 1.7±0.2 pM, n=6, P<0.05) **(Figure 6.14D)**. No statistical change to serosal ACh was evident in bladders that had been pre-treated with the vehicle when compared to either RTX or saline **(Figure 6.14D)**.

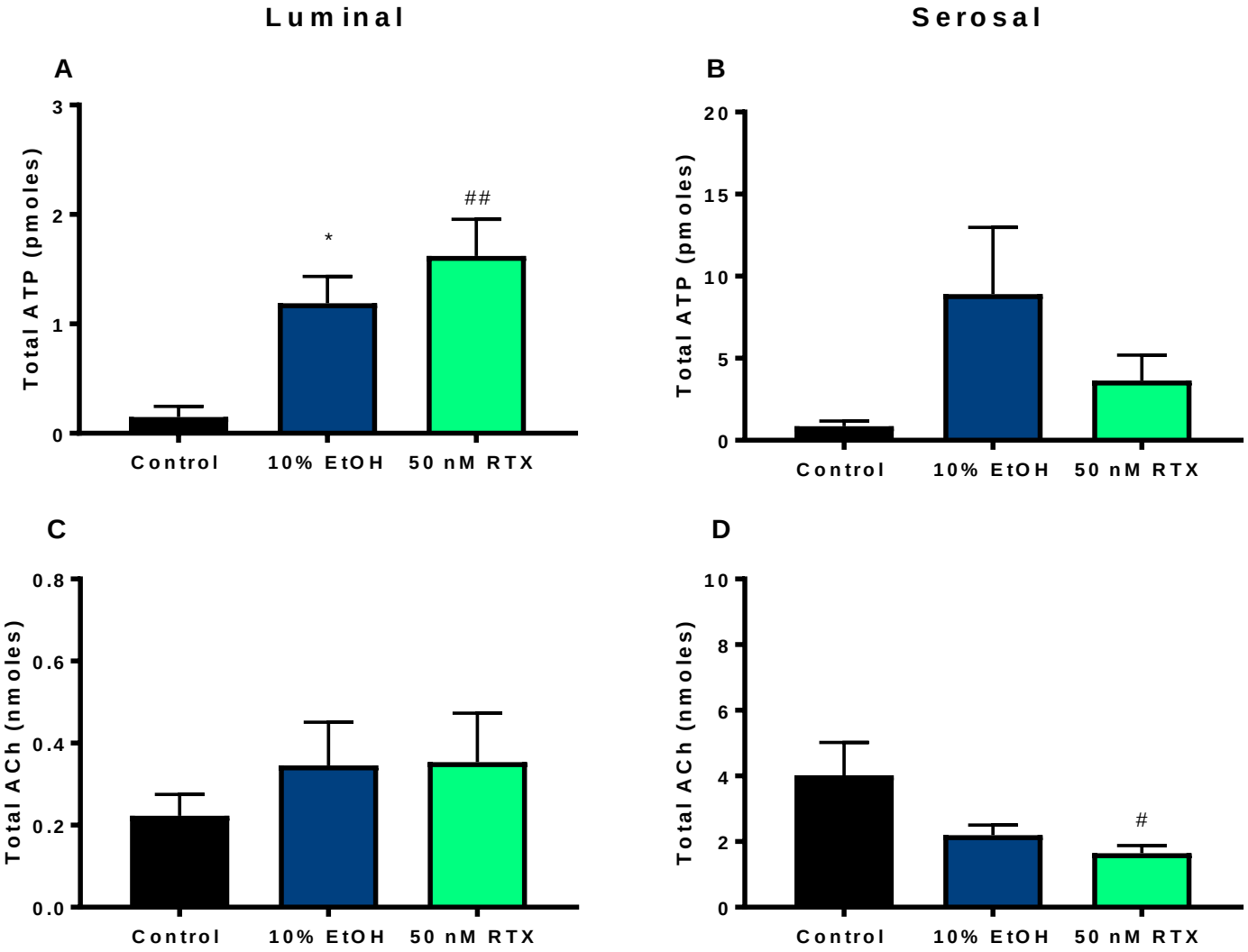

Figure 6.14: Amounts of ATP (in pmoles) and ACh (in nmoles) released from the luminal (A&C) and serosal surfaces (B&D) of whole bladders. Data is represented as mean ± SEM analysed by one-way ANOVA with Tukey-Kramer multiple comparisons post-hoc test (n≥5), (*P<0.05 saline vs. 10% EtOH, #P<0.05, ##P<0.01 saline vs. 50 nM RTX).

The contractile responses to ATP and KCl

To determine if intravesical treatment 24-hours prior had affected the contractile responses of the bladder, ATP (1 mM) and α,β m-ATP(10 μM) assessed the purinergic responses and KCl (60 mM) assessed the non-receptor mediated responses.

The non-receptor mediated contractile response of RTX pre-treated bladders to KCl was significantly enhanced when compared to saline pre-treated bladders (saline 13.6±2 mmHg vs. 50 nM RTX 22.3±1.8 mmHg, n=6, P<0.01) **(Figure 6.15)**. No statistically significant change in response to KCl was detected between bladders that had been pre-treated with the ethanol vehicle when compared to either saline or RTX pre-treated bladders **(Figure 6.15)**. The following responses to ATP, α,β m-ATP, cumulative concentration-curves to carbachol and EFS were additionally normalised to KCl to discriminate between receptor and non-receptor mediated changes.

The contractile response to ATP was significantly enhanced by 100% in the ethanol vehicle and RTX treated bladders when compared to saline. **(Figure 6.16A)**. The response did not vary between bladders pre-treated with the vehicle or RTX **(Figure 6.16A)**. When normalised to KCl, the previous augmentations to ATP found after pre-treatment with the ethanol vehicle and RTX was now only significant in bladders that had been pre-treated with RTX when compared to saline (saline 35.6±6.3 mmHg vs. 50 nM RTX 95.1±17.3 mmHg, n=6, P<0.01). No statistical change could be found in bladders pre-treated with the ethanol vehicle when they were compared to either saline or RTX **(Figure 6.16B)**.

Similarly, the initial responses to α,β m-ATP were significantly increased by 100% in the ethanol vehicle and RTX pre-treated groups compared to the saline group **(Figure 6.16C)**. When normalised to KCl, the response remained significantly elevated after pre-treatment with RTX when compared to saline (saline 57.3±6.6mmHg vs. 50 nM RTX 119.5±24.8 mmHg, n=6, P<0.05) while no statistical change could be found in bladders treated with the ethanol vehicle when they were compared to either saline or RTX pre-treated bladders **(Figure 6.16D)**.

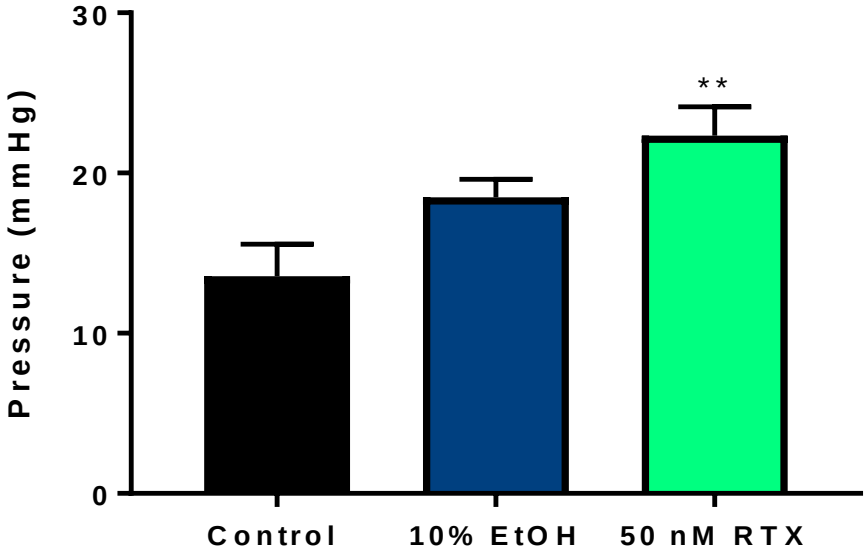

Figure 6.15: The responses of mouse bladders to 60 mM KCl 24-hours after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX. Data is represented as mean ± SEM analysed by one-way ANOVA with Bonferroni multiple comparisons post-hoc test (n=6), (P<0.01 saline vs. 50 nM RTX).**

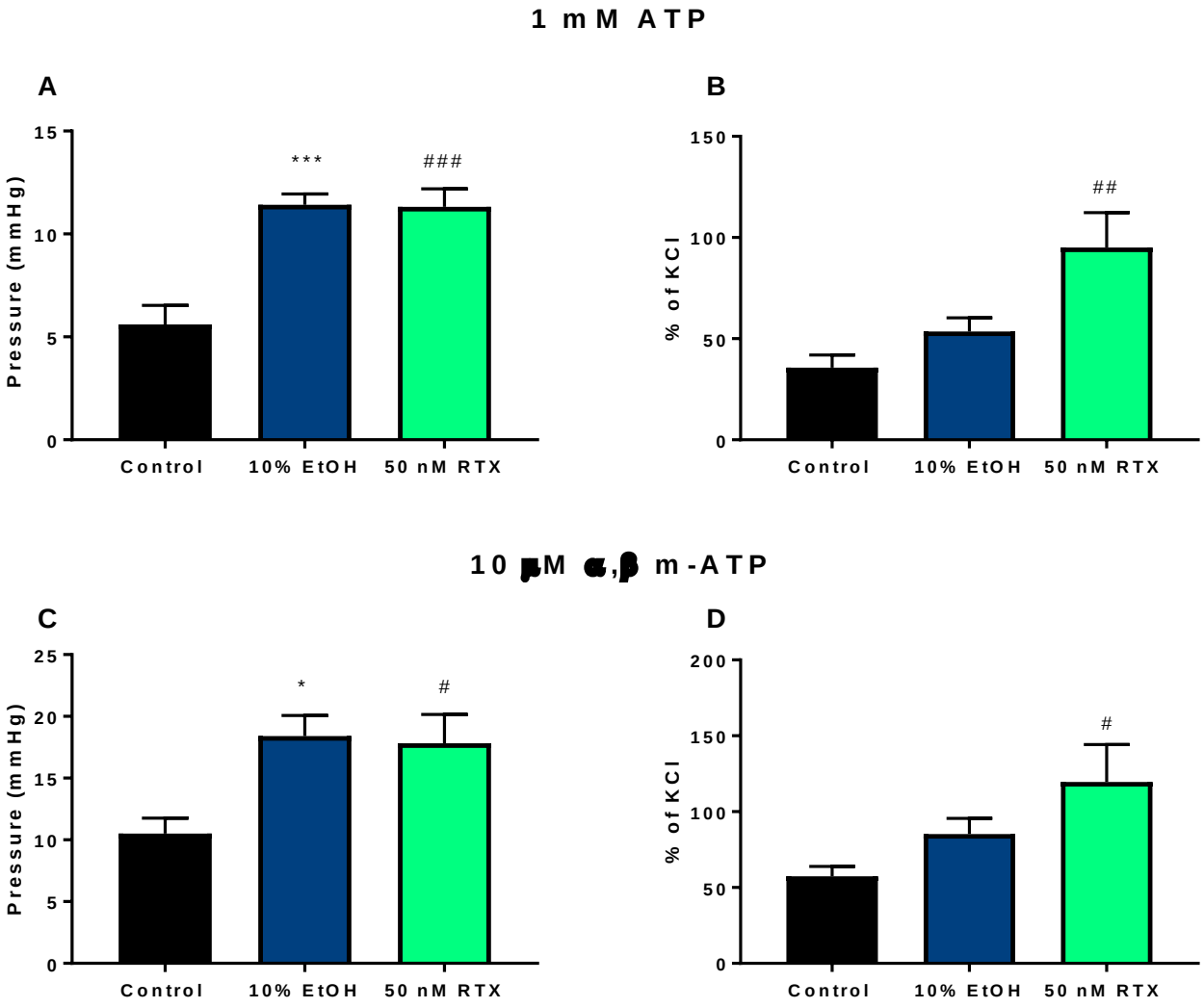

Figure 6.16: The effect of 1 mM ATP (A,B) and 10 µM α,β m-ATP (C, D) on contractile pressures (A,C) in bladders 24-hours after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX and normalised to KCl (B,D). Data is represented as mean ± SEM analysed by one-way ANOVA with Bonferroni multiple comparisons post-hoc test (n≥5), (*P<0.05, ***P<0.001 saline vs. 10% EtOH, #P<0.05, ##P<0.01, ###P<0.001 saline vs. 50 nM RTX).

The responses to muscarinic and adrenergic stimulation

The contractile responses to cumulative concentrations of carbachol as well as the relaxation response to cumulative concentrations of isoprenaline were investigated in mouse bladders 24-hours after intravesical treatments. **Figure 6.17** shows a recorded trace of the mouse bladder responding to cumulative concentrations of carbachol **(A)** and the relaxation response achieved to cumulative concentrations of isoprenaline against passive tension **(B)**.

For bladders that had been pre-treated with the ethanol vehicle or RTX, the maximal response to carbachol was significantly enhanced when compared to saline while there was no change to the pEC_{50} values between any of the groups **(Figure 16.18A, Table 6.1)**. There was no further alteration to the response in bladders pre-treated with the vehicle and RTX **(Figure 16.18A, Table 6.1)**. When all the responses were normalised to KCl, the significant enhancements observed in bladders pre-treated with the ethanol vehicle were no longer evident but the maximal response to carbachol remained significantly greater in RTX pre-treated bladders compared to saline. **(Figure 16.18B, Table 6.1)**.

Cumulative concentration-response curves to isoprenaline were similar for all experimental groups and neither pEC_{50} values nor maximum responses were altered in any of the treatment groups (**Figure 6.18C, Table 6.1)**.

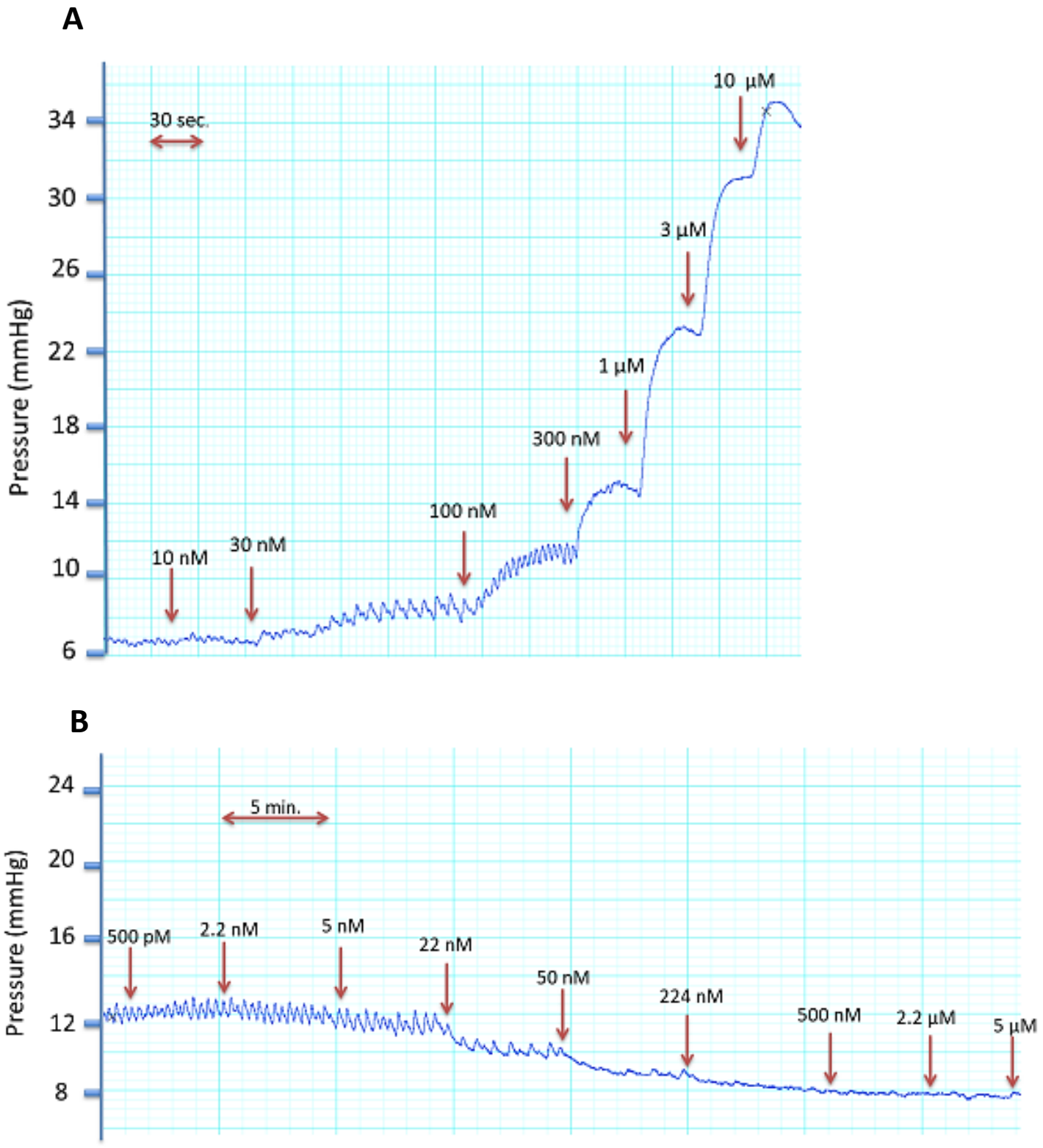

Figure 6.17: Illustration of the cumulative effects of carbachol on contraction (A) and the cumulative effects of isoprenaline on relaxation (B) in the mouse bladder.

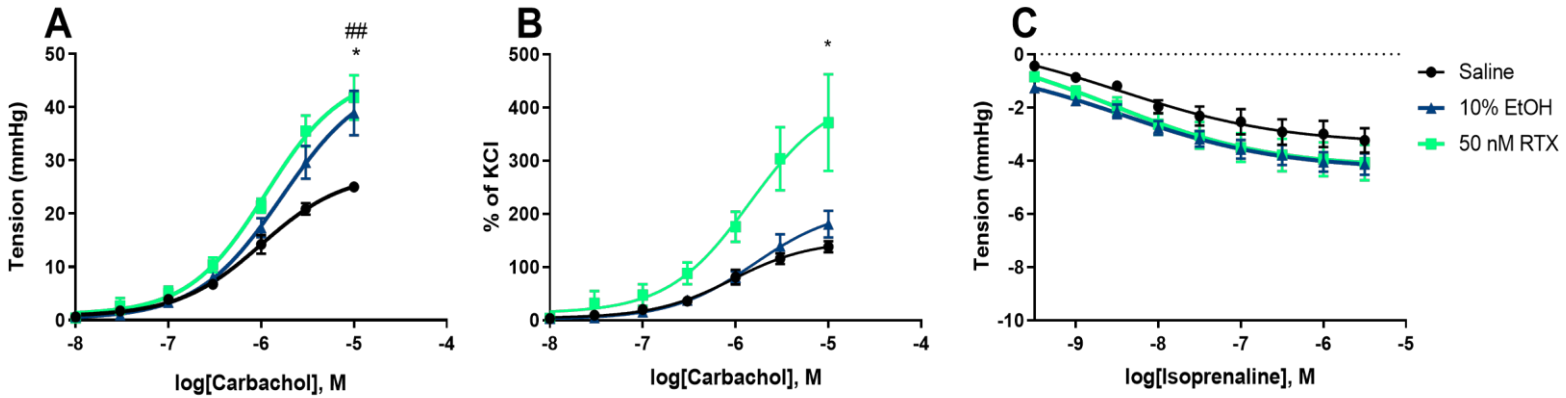

Figure 6.18: A- The response to cumulative concentrations of carbachol, B- after normalization to KCl and to C-cumulative concentrations of isoprenaline in bladders 24-hours after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX. Data is represented as mean ± SEM analysed with multiple comparison F-test and one-way ANOVA using Tukey-Kramer multiple comparisons post-hoc test (n=6), (*P<0.05 saline vs. 10% EtOH, #P<0.05, ##P<0.01 saline vs. 50 nM RTX).

Table 6.1: Mean (± SEM), maximum responses and pEC_{50} values for carbachol and isoprenaline. Data is represented as mean ± SEM analysed with multiple comparison F-test and one-way ANOVA using Tukey-Kramer multiple comparisons post-hoc test (n=6), (*P<0.05 saline vs. 10% EtOH, #P<0.05, ##P<0.01 saline vs. 50 nM RTX).

Carbachol	Control (0.9% saline)	10% EtOH	50 nM RTX
pEC_{50} (± SEM)	6.00±0.11	5.78±0.18	5.95±0.12
Maximum response (mN)	27.71±2.49	46.11±7.45*	46.47±4.99##
	Normalised to KCl		
pEC_{50} (± SEM)	6.03±0.15	5.79±0.24	5.85±0.35
Maximum response/% of KCl	149.6±18.65	211.8±45.49	428.1±130.7#
Isoprenaline			
pEC_{50} (± SEM)	8.46±1.65	8.42±0.98	8.54±1.51
Maximum response (mN)	-3.38±0.79	-4.33±0.58	-4.26±0.83

Responses to electrical field stimulation

Electrical field stimulation was used to assess if any changes had occurred to nerve-mediated contraction 24-hours after intravesical treatment. Additionally, the effects of the NOS inhibitor L-NNA (100 µM), atropine (1 µM) and α,β m-ATP (10 µM) were added to the isolated bladders in the organ bath without washout and assessed at 20 Hz stimulation to investigate the contributions made by NO, muscarinic and P2X receptors at this frequency. **Figure 6.19A** demonstrates the typical pressure response to nerve-mediated contractions at 1-20 Hz in the whole mouse bladder while **Figure 6.19B** shows the effect of pharmacological agents on nerve-mediated contractions at 20 Hz.

For mouse bladders that had been pre-treated with the ethanol vehicle or RTX, the responses to nerve-mediated stimulation were significantly enhanced for all frequencies tested compared to saline pre-treatment **(Figure 6.20A).** There was no change to the responses between the vehicle and RTX pre-treated bladders **(Figure 6.20A)**. Likewise, following normalization to KCl, the response of bladders that had been pre-treated with the vehicle or RTX also remained significantly enhanced compared to the saline pre-treated bladders with no additional changes between the vehicle or RTX. **(Figure 6.20B, Table 6.2)**.

The addition of L-NNA and atropine did not significantly change the responses to EFS at 20Hz stimulation for either saline or vehicle pre-treated bladders. However, further desensitization of the P2X receptors with α,β m-ATP significantly knocked down the responses in these bladders by 82% and 85% respectively demonstrating the ATP is the predominant neurotransmitter **(Figure 6.21A,B)**. For bladders that had been pre-treated with RTX, L-NNA did not statistically change the response to EFS at 20 HZ, whereas the addition of atropine significantly reduced the response by 30% and was reduced by a further 57% in the presence of α,β m-ATP indicating that neural ACh plays a larger role after intravesical pre-treatment with RTX **(Figure 6.21C)**.

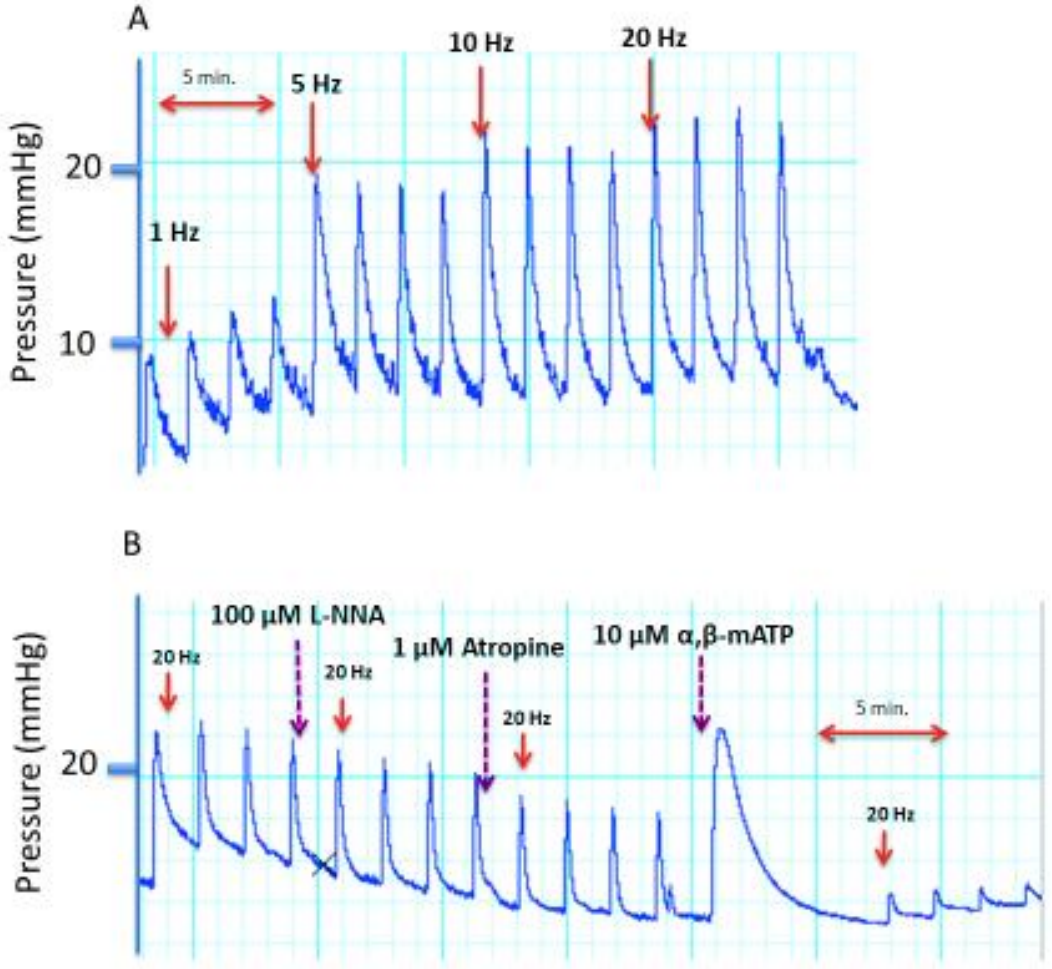

Figure 6.19: A- Demonstrative trace of the contractions that occur in mouse bladders in response to EFS at 1,5,10 and 20 Hz. B- After an additional 4 spikes of stimulation with 20 Hz, 100 µM L-NNA, 1 µM of atropine and 10 µM of α,β m-ATP were added to the organ bath to assess the various contributors to nerve-mediation contraction.

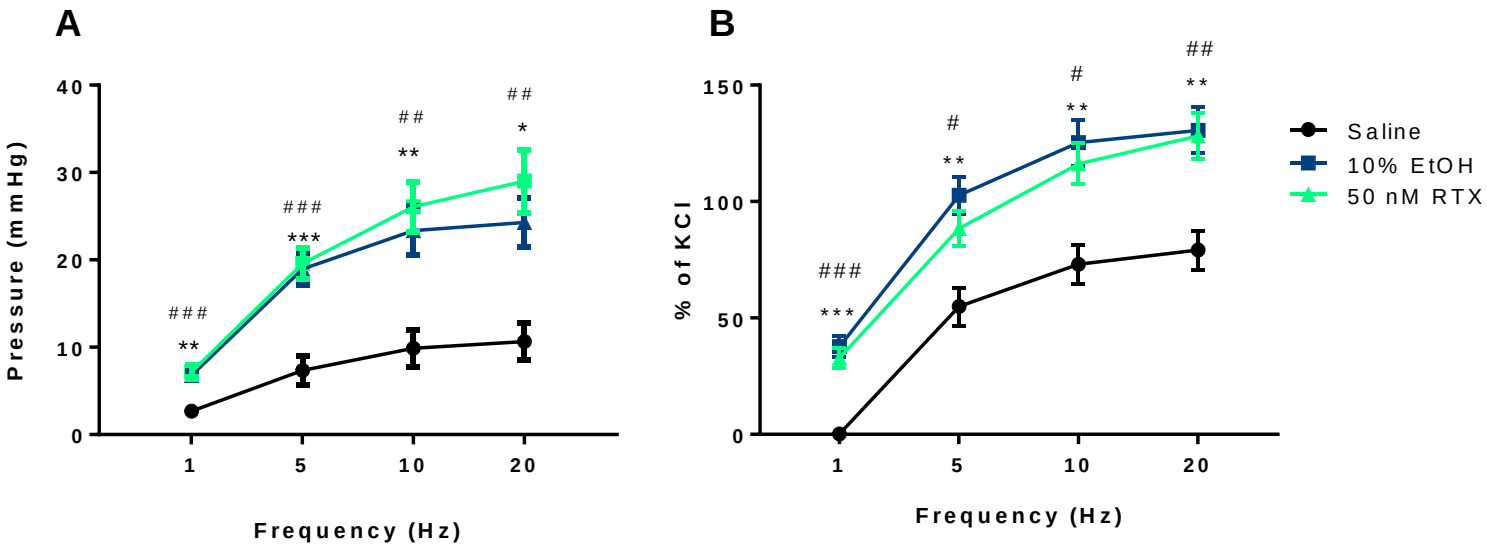

Figure 6.20: A- The responses to EFS (50V, 0.2 ms pulse duration) delivered as a 5-second train every 100 ms at 1,5,10 and 20 Hz and B- responses to EFS normalised to KCl, 24-hours after intravesical treatment with 0.9% saline (control), 10% EtOH (vehicle control) or 50 nM RTX. Data is represented as mean ± SEM analysed by one-way ANOVA with Bonferroni multiple comparisons post-hoc test (n=6), (*P<0.05, **P<0.01, ***P<0.001 control vs 10% EtOH, #P<0.05, ## P<0.01, ###P<0.001 control vs 50 nM RTX).

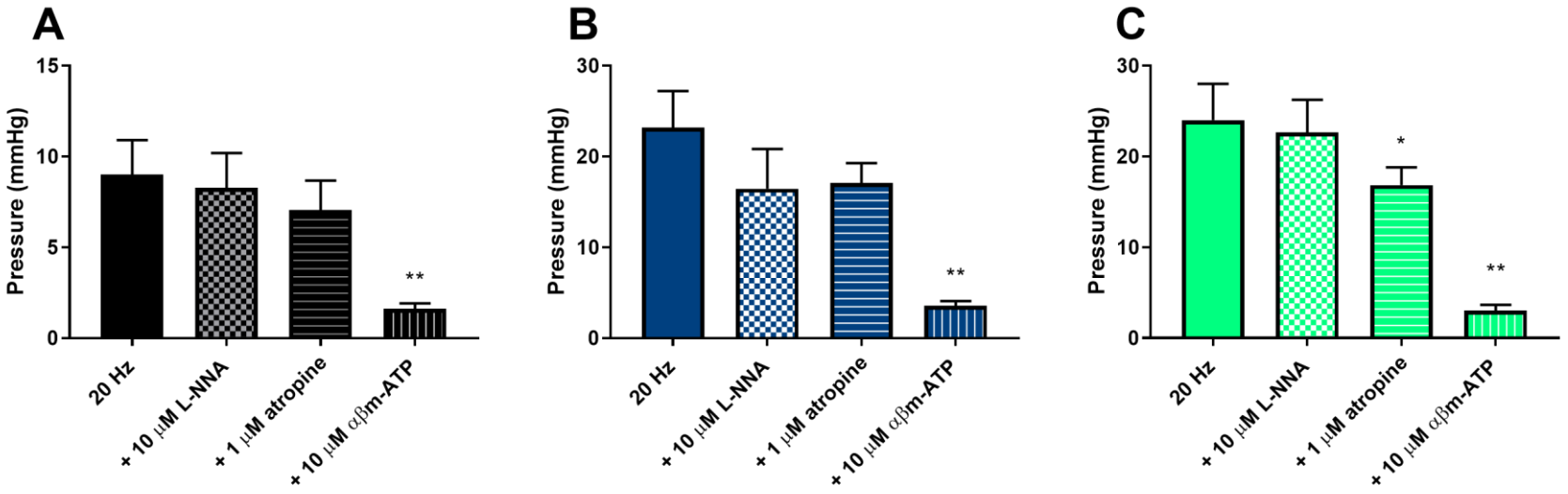

Figure 6.21: The response to EFS (50V, 0.1 ms delay, 0.2 ms pulse duration) delivered as a 5-second train every 100 ms at 20 Hz in the presence of L-NNA, Atropine and α,β m-ATP 24-hours after intravesical treatment with 0.9% saline (control) (A), 10% EtOH (vehicle control) (B) or 50 nM RTX (C). Data is represented as mean ± SEM analysed by one-way ANOVA with Dunnet compare all vs control multiple comparisons post-hoc test (n≥5), (*P<0.05, **P<0.01 20 Hz vs all)

6.5 Discussion

The previous chapter focused on the effects of luminally applied resiniferatoxin (RTX) and its vehicle (10% ethanol) on isolated strips of pig bladder tissue. The pig bladder represents a suitable model in which the findings can be extrapolated to the human bladder with a fair degree of confidence. However, these results are presented on one plane and have not taken into consideration how RTX and its vehicle may affect whole bladder functioning. Owing to its more potent desensitizing nature and better tolerability compared to capsaicin, RTX could potentially be a useful tool for long-term improvement in pain and symptoms associated with IC/BPS. To better understand the actions of RTX on the whole bladder as a unit, this study has chosen a mouse model that has been suggested to be closer to human bladder than any other rodent species (Andersson et al., 2011). The physiologically thinner urothelium and more dominant purinergic neurotransmission found in the mouse bladder may allow some insight into human bladder pathology where urothelial thinning and more active purinergic neurotransmission becomes characteristic, especially in IC/BPS (Phillips and Davies, 1980, Palea et al., 1993, Slobodov et al., 2004, Fabiyi and Brading, 2006).

The general effects of treatments

Typically, all intravesical treatments given to animals were well tolerated with no apparent differences in wellbeing including behaviour and appearance. This is consistent with other studies that have found similar spinal *c-fos* immunoreactivity to catheterization alone and instillation of 10% ethanol for 30-minutes into the rat bladder. In the same study, intravesical instillation with RTX (in 10% ethanol) was found to be mildly irritating in comparison to intravesical capsaicin (Avelino et al., 1999, Avelino et al., 1998). Furthermore, Ishizuka et al. (1995b) also observed normal behavior and no signs of distress apart from occasional lower abdomen licking after intravesical instillation of 30 nM RTX. This is in line with intravesical RTX investigations in humans that reported treatment is tolerable (Lazzeri et al., 2000, Apostolidis et al., 2006). There were also no changes to body weight between the groups before and after treatment and no differences in water intake in the 24-hours post-treatment period.

Voiding pattern analysis

The voiding behaviour of mice was observed immediately before and 24-hours after intravesical treatment. Control C57BL/6J mice in other studies have been found to urinate repeatedly in 1 to 2 locations suggesting sanitary conduct, conscious preference and continence (Kanasaki et al., 2013, Yu et al., 2014). Neither pre-treatment with saline nor with the vehicle ethanol interfered with the voiding behaviours in mice for this observation. However, mice that had been pre-treated with RTX developed an overactive bladder phenotype with a two-fold increase in the number of voiding events. Further spot analysis revealed that this increase in voiding frequency following RTX pre-treatment was associated with a decrease in the volume of primary voids and an increase in the number of small voids. It has been observed in rat bladders 2-hours after intravesical instillation with 100 nM RTX, there was a significant enhancement in spinal *c-fos* expression in the lamina I and intermediolateral grey matter. This was, as mentioned earlier, suggested to be mildly irritating and may explain the enhanced frequency of voiding events that was found in RTX treated mice for this study (Avelino et al., 1999). On the other hand, Janssen et al. (2016) observed a decrease in spinal *c-fos* expression 24-hours after intravesical treatment, although, 1 nM RTX was used in contrast to 50 nM for this study is likely to be more irritating to the bladder. Furthermore, as RTX targets and desensitizes the TRPV1 channels, TRPV1 knock out (KO) mice were found to void lower volumes of urine more frequently which is similar to the findings of this study (Yoshiyama et al., 2015).

Bladder compliance and spontaneous activity

Overall, bladder compliance was not altered in all treatment groups which was not unexpected as it was found in TRPV1 KO mice that capacity was unchanged compared to its wildtype controls (Grundy et al., 2018b). Additionally, for normal rats, bladder capacity remained unchanged 24-hours after intravesical administration of 10% ethanol (Ost et al., 2003).

Similar to other species, spontaneous activity occurs in the mouse bladder (Meng et al., 2008). The amplitude of spontaneous contractions was enhanced by 90% in bladders that were pre-treated with the ethanol vehicle. For these bladders that were pre-treated with ethanol, some of them had quite pronounced spontaneous contractions while others did not and the larger SEM reflects that. The mechanisms and components of spontaneous activity in the bladder are

complex, and in the mouse bladder, it has been found that pharmacological blockade of the big potassium (BK) channels resulted in an elevation in amplitude of spontaneous phasic contractions of mouse detrusor strips while frequency was decreased (Petkov et al., 2001). In other tissues, ethanol has been shown to inhibit BK channel activity, and inhibition of this channel by ethanol in the bladder may be a contributing factor to the increased amplitude of spontaneous activity found in this observation (Walters et al., 2000).

Although frequency remained similar between all experimental groups, blockade of BK receptors in mouse detrusor reduced spontaneous contraction frequency (Petkov et al., 2001). As this is not the case for bladders pre-treated with the ethanol vehicle, it would suggest the presence of other excitatory mediators associated with ethanol use, such as prostaglandins or substance P (SP) which are known to enhance spontaneous frequency and may be "offsetting" any possible reduction (Collier et al., 1975, Callahan and Creed, 1986, Maggi et al., 1990b, Trevisani et al., 2002, Kobayter et al., 2012).

Effects of intravesical treatment on mediator release

Distension of mice bladders in response to filling with saline solution results in liberation of ATP and ACh from both the luminal and serosal surfaces of the whole bladder (Collins et al., 2013, Grundy et al., 2018b). Studies have shown that the dominant source of ATP found in the bladder is from the urothelium/lamina propria and it diffuses to both the luminal and basolateral sides (Kumar et al., 2004, Lewis and Lewis, 2006). Additionally, Yoshida et al. (2006) identified that the urothelium/lamina propria supplies a large proportion of stretch-induced non-neuronal ACh to the bladder (Yoshida et al., 2006). The urothelium forms a barrier between the luminal contents and the underlying tissue. It is thought that ATP and ACh released from the luminal side of the urothelium/lamina propria interacts with the purinergic and muscarinic receptors found on the lumen while ATP and ACh released from the basolateral surface interacts with the same receptors found on the underlying cells and nerves (Chopra et al., 2008, Vlaskovska et al., 2001, Hanna-Mitchell et al., 2007, Yu and de Groat, 2010, Birder and Andersson, 2013). Other potential contributors to distension induced serosal ATP and ACh include tension on detrusor cells and spontaneous releases from bladder nerves (Young et al., 2008, Zagorodnyuk et al., 2009b, Cheng et al., 2011b). Accordingly, samples were taken from the luminal and serosal surfaces of the mouse bladder 24-hours after intravesical treatment to examine ATP and ACh release at distension pressures of 15 mmHg that is reported to be the

micturition threshold in mice (Daly et al., 2007).

ATP

For bladders that had been pre-treated with RTX and the ethanol vehicle, the distension induced release of luminal ATP was increased by eleven-fold and eight-fold respectively. The alteration between RTX or ethanol was not statistically significant, indicating that ethanol mediates this enhancement. The reason behind the elevation found in distension induced ATP may be due to the stimulatory effect of the remaining ethanol on the TRPV1 channels found within the urothelium which are less sensitive to desensitization compared to the TRPV1 channels found on the nerves (Birder et al., 2001, Trevisani et al., 2002). Mice devoid of TRPV1 channels were unable to produce stretch-induced ATP (Grundy et al., 2018b). Moreover, cultured urothelial cells from TRPV1 knockout mice produced less hypotonically induced ATP release (Birder et al., 2002). For these experiments, as there is distension induced luminal ATP release after treatment with ethanol and RTX, this would suggest the presence of a urothelial cell layer. Pig urothelium/lamina propria in chapter four and five was unable to produce any stretch-based ATP release after the removal of the urothelial cell layers by 30% ethanol. That's not to say that ethanol's actions have not damaged any of the upper layers of the urothelium as urothelial injury has also been found to enhance luminal ATP in rabbit and rat bladders. (Tapani et al., 1996, Smith et al., 2005, Lewis and Lewis, 2006). However, it could be assumed that the majority remains. For this study, the consequence of enhanced luminal ATP is unclear. Evidence would suggest an increase in bladder sensory activity which would perhaps be evident in the VPA for ethanol and RTX treated mice. However, only mice treated with RTX were found to have an increase in voiding frequency (Vlaskovska et al., 2001). Also, mice given RTX by intraperitoneal injection had significantly enhanced endogenous ATP and P2X3 immunoreactivity in DRG neurons seven days after treatment (Lin et al., 2013). An RTX mediated increase in P2X3 receptors on sensory nerves may be another explanation for increased urination frequency found in these mice for this study.

The levels of serosal ATP for this experiment remained unchanged between all experimental groups which were similar to the observations made by Smith et al. (2005) who identified an increase in ATP on the urothelial side of the bladder but not the serosal side after treating rats with the bladder irritant cyclophosphamide.

ACh

Distension induced luminal ACh release and serosal ACh remained similar in all experimental groups except for mice pre-treated with RTX in which serosal ACh was reduced by 60%. A potential contributor to serosal ACh has been suggested to arise from spontaneous release from nerves within the bladder wall (Young et al., 2008, Zagorodnyuk et al., 2009b). In the guinea pig bladder, spontaneous ACh release was found to be from nerve fibres that were TTX insensitive and further blockade of neuronal N-type, P/Q-type and R-type Ca^{2+} channels did not affect physostigmine (cholinesterase inhibitor) evoked contractility but significantly decreased EFS-induced contractions by approximately 60% (Zagorodnyuk et al., 2009b). Furthermore, in sections of pig bladder tissue that had been treated with 500nM RTX, there was found to be a decrease in nerve fibres immunoreactive to vesicular acetylcholine transporters (vACHt) in the bladder wall seven days after treatment (Lepiarczyk et al., 2017). The reduction found in serosal ACh for this study may be due to degeneration and neurotoxicity of a subset of vACHt fibres in response to RTX. Diminished ACh levels may also reflect desensitization and depletion of neuropeptides from sensory nerves as SP has been shown to evoke TTX insensitive ACh release from the guinea pig bladder (Shirakawa et al., 1989). Less available ACh in RTX treated bladders may be allowing the more prominent luminal ATP release to have a greater influence on voiding activity (Pandita and Andersson, 2002). Moreover, it was found in the mouse bladder that application of muscarinic agonist into the organ bath depressed sensory nerve activity on bladder filling (Daly et al., 2014). Hence, the depressed levels of serosal ACh found in this study could also be enhancing bladder sensation on bladder filling contributing to the increased voiding activity found in these mice.

Contraction/relaxation analysis

Based on the initial findings of this study, the vehicle ethanol has increased the amplitude of spontaneous contractions and luminal ATP release. Resiniferatoxin, on the other hand, has increased voiding frequency, luminal ATP release and has decreased serosal ACh levels. The effect of treatment on other structures and reflexes of the bladder is unknown. Therefore, the contractile/relaxation functions were compared in bladders 24-hours after intravesical treatment.

Response to ATP and KCl

A two-fold enhancement in the contractile response to non-receptor mediated stimulation (KCl) was found in RTX pre-treated bladders. This enhancement in response to KCl could reflect the action of ethanol and RTX activating TRPV1 channels on TRPV1 positive cells. Activation of TRPV1 enhances Ca^{2+} influx and releases neuropeptides, therefore, the already enhanced levels of intracellular Ca^{2+} would be more sensitive to the depolarizing actions of KCl (Karaki et al., 1984, Winter et al., 1990, Trevisani et al., 2002). Additionally, it has been found in the rat gastric mucosa that the damage produced by the administration of 96% ethanol was potentiated when it was co-administered with RTX (0.6 and 1.0 µg/kg) (Abdel-Salam et al., 1995). In the mouse bladder, RTX may have slightly potentiated the effects of ethanol allowing for unhindered diffusion of potassium (K^+) to the underlying bladder structures enhancing the overall contractile response.

The response to purinergic stimulation by both ATP and α,β m-ATP was also enhanced by 100% in bladders pre-treated with the ethanol vehicle or RTX. However, when normalised to KCl, it was only the purinergic response after RTX that remained enhanced suggesting an alteration has occurred to purinergic signalling or perhaps the way ATP is hydrolyzed as a result of RTX treatment. Since the desensitization process in response to RTX occurs over a period of days, excitatory mediators dispersed in the tissues could also be contributing to this enhanced response (Szallasi et al., 1989). Also, ATP has been found to stimulate prostaglandin release from the urothelium of rat bladders. (Pinna et al., 2000). As luminal ATP release in our study was already enhanced by RTX pre-treatment, ATP could be promoting an increase in prostaglandin production in addition to possible enhancements produced by the ethanol vehicle (Collier et al., 1975). Interestingly, prostaglandins have also been found to augment the contractile responses of ATP (Husted et al., 1980).

Contractile/relaxation response to carbachol and isoprenaline

The responses to muscarinic stimulation were enhanced after intravesical pre-treatment with the ethanol vehicle or RTX. As the difference between the enhanced muscarinic responses was unchanged between ethanol or RTX, this indicates that ethanol is once again behind the enhancement. Once the responses had been normalised to KCl, only bladders that had been treated with RTX still had enhanced responses to muscarinic stimulation and demonstrates that the enhancements made by ethanol are mediated by non-receptor sensitivity rather than

interference of the muscarinic receptors. This is supported by Trevisani et al. (2002), who found that ethanol did not enhance the Ca^{2+} response to carbachol but rather enhanced intracellular Ca^{2+} via TRPV1 channel activation. The persisting enhancement by RTX could indicate a number of things including the modification to muscarinic receptor signalling, possible stimulation of the Rho kinase (ROCK) and protein kinase C (PKC) pathway or an alteration to UDIF release. Resiniferatoxin is not traditionally associated with alterations to the ROCK/PKC pathway in the smooth muscle, consequently, information is scarce. However, muscarinic responses are attenuated by both the ROCK inhibitor Y-27632 and the PKC inhibitor GF-109203X (Shahab et al., 2012). Stimulation or delayed decay of either pathway by RTX could lead to enhanced intracellular Ca^{2+} potentially amplifying the response to muscarinic stimulation. Additionally, the presence of the urothelium in the mouse bladder reduces the contractile response to carbachol which is thought to be mediated by UDIF (Canda et al., 2009). Moreover, an abstract published by Scott et al. (2005) demonstrated that M2 receptors are involved in its release. As it was assumed in this study that the majority of the urothelium is present after all intravesical treatment, RTX may have damaged the cells/receptors responsible for producing UDIF leading to the enhanced muscarinic responses.

The mouse bladder relaxes via β-adrenoceptor stimulation (Canda et al., 2009). For all experimental groups in this study, there were no changes found to this relaxation response after β-adrenoceptor stimulation confirming that RTX and its vehicle do not interfere with sympathetic-mediated pathways.

Electrical field stimulation

Bladders that had been pre-treated with the ethanol vehicle or RTX produced greater responses to all frequencies of nerve stimulation. The enhanced responses persisted after normalization to KCl suggesting an alteration has occurred to transmitters or receptors involved in the parasympathetic pathway. As mentioned in previous chapters, there is evidence that ethanol induces the release of prostaglandins, which are also released in response to injury and inflammation (Collier et al., 1975, Maggi, 1992, Neuman et al., 2002). It has been shown in rabbit detrusor that prostaglandins (PGE_2 and $PGF_{2\alpha}$) augment the responses to EFS via non-adrenergic, non-cholinergic (NANC) mechanisms and also increase the initial rapid phasic response of the tissue to ATP by 123% (Husted et al., 1980). Prostaglandins also contribute to an enhanced response to EFS in rat and human detrusor strips while their intravesical

application has been suggested to promote the release of SP and neurokinin A (NKA) from nerves below the urothelium in the rat bladder (Larsson, 1980, Ishizuka et al., 1995a). Substance P and SP containing fibres have been detected throughout the mouse bladder including the sub-urothelial space (Maggi et al., 1987b, Mitsui and Hashitani, 2013) Moreover, SP on rat bladders has been shown to enhance the amplitude of TTX sensitive distension induced rhythmic contractions (Maggi et al., 1984). However, another study by Maggi et al. (1987a) found no correlation between SP levels and the response to field stimulation which was similar to the findings of Callahan and Creed (1986). The present results from this study support the idea that an increase in prostaglandin production as a result of ethanol may contribute to the enhanced response to EFS either directly or indirectly by its interaction with ATP.

Examination of the various contributors to nerve-mediated contraction revealed that ATP is the dominant neurotransmitter in the mouse bladder, blocking the majority of the response to EFS when α,β m_ATP was added to the organ bath in addition to the NOS inhibitor L-NNA and muscarinic antagonist atropine. Blockade of NOS including antagonism of muscarinic receptors did not alter the responses to EFS for bladders in the saline and ethanol vehicle groups. However, for bladders pre-treated with RTX, although the nerve-mediated response was unchanged by inhibiting NOS, the additional antagonism of the muscarinic receptors reduced the response by 30% indicating that neuronal ACh plays a more significant role in these bladders. As the urothelium has not been removed, the reason behind these enhanced responses could be similar to the reason behind the enhanced responses to muscarinic stimulation which was a reduction in UDIF, since UDIF also reduces the responses to EFS in intact bladder strips (Propping et al., 2013).

As RTX desensitizes sensory nerves, some neuropeptides including NKA have been shown to enhance ACh release from nerve terminals in the guinea pig bladder and pituitary adenylate cyclase activating polypeptide (PACAP) in the rat hippocampus (Shinkai et al., 1991, Masuo et al., 1993). Therefore, specific neuropeptides may be contributing to the stronger ACh component to nerve-mediated stimulation in RTX pre-treated bladders. Also, PACAP potentiates the responses to EFS in rat bladders (Braas et al., 2006, Herrera et al., 2006) Furthermore, atropine has been found to inhibit the facilitatory effects of PACAP in other tissues suggesting an interaction between peptidergic and cholinergic transmission exists (Roberto and Brunelli, 2000).

Implications of using murine bladder

The findings of this study examining the intravesical instillation of saline, ethanol and RTX in the mouse bladder have produced some additional results and a few similarities with what we obtained in previous chapters. The similarities found between the pig and mouse either immediately or 24-hours after treatment (respectively) with ethanol or RTX include no change to urothelial stretch/distension induced ACh, enhanced amplitude of spontaneous contractions after treatment with the ethanol vehicle, no alterations to the frequency of spontaneous contractions and no interference to adrenoceptor-mediated relaxation. For the new additional results found in the mouse bladder 24-hours after intravesical treatment, it was important to discern if non-receptor or receptor-mediated mechanisms contributed to the enhancements found in bladders treated with the ethanol vehicle or RTX to purinergic stimulation, muscarinic stimulation and EFS. Once non-receptor mediated sensitivity had been accounted for, this uncovered some unique alterations to receptor-mediated function by RTX pre-treatment. It is not certain if they are due solely to RTX or RTX combined with its vehicle, as RTX has been found to potentiate the effects of ethanol and together they may be working in synergy (Abdel-Salam et al., 1995). The unique effects of RTX consisted of amplification of the purinergic and muscarinic responses whereas the enhancements made by ethanol were due to non-receptor mediated sensitivity. Interestingly and possibly related to these changes was the development of an overactive bladder phenotype after RTX pre-treatment.

The overactive phenotype that increases the voiding frequency after RTX pre-treatment is suspected to occur by the previously mentioned enhancements found in purinergic and muscarinic responses as well as the previously reported enhancements in spinal *c-fos* and P2X receptors in DRG neurons after RTX exposure (Avelino et al., 1999, Lin et al., 2013). Other contributing factors from this study may be due to the lowered levels of ACh found in the serosal fluid allowing the excess luminal ATP to become more prominent in its signalling pathway.

The new and additional findings mentioned above after pre-treatment with the ethanol vehicle and RTX could be due to several factors which include an enhanced binding affinity of RTX in the mouse when compared to the pig and the time after treatment when the experiments were carried out (the immediate effects in the pig versus 24-hours later in the mouse) (Szallasi and Blumberg, 1990, Szallasi et al., 1993). Another causative factor could be related to the

pharmacology of RTX which has a slower onset of action (Winter et al., 1990). Other experiments in the mouse bladder concerning RTX have also identified noteworthy results 24-hours after intravesical treatment such as a decrease in spinal *c-fos* expression and an increase to the sensory threshold in C-fibres (Bicer et al., 2014, Janssen et al., 2016). Other aspects to consider when interpreting these results is difference in the volume/pressure relationship of the whole bladder versus tension generated by bladder strips and the anatomical and physiological differences between the mouse and pig bladder. As the urothelium is the first point of contact for intravesical delivery, its structure is important. The pig bladder urothelium is made up of several urothelial layers compared to the mouse urothelium which is reportedly made up of three cell layers which would make it an easier barrier to overcome (Cheng et al., 2011b, Phillips and Davies, 1980). In chapter four and five, it did not appear that the 10% ethanol vehicle and RTX had diffused past the urothelium/lamina in the pig since they had no effect. However, this does not appear to be the case for this study as the ethanol vehicle and RTX have both overcome the urothelial cell layer and have affected efferent activities of the bladder either directly or indirectly. There is evidence to support that a considerable portion of the altered efferent activity may be indirect via ethanol and RTX activation of TRPV1 channels on the urothelium and afferent nerves which causes the release of excitatory neuropeptides, as well as by ethanol-induced prostaglandin production (Birder et al., 2001, Trevisani et al., 2002, Grundy et al., 2018b, Collier et al., 1975). Overall, these results demonstrate the many side effects associated with the ethanol vehicle and do not entirely reflect the actions of RTX in the bladder which was similar to what was found in the pig bladder in chapter five. This is in agreement with Ost et al. (2003) that ethanol is not an inert vehicle.

All of the observed enhancements to bladder activity following treatment with RTX and its ethanol vehicle fit with the voiding analysis of this study and patient experience from previous studies which will now be discussed in greater detail in the general discussion.

Summary

In contrast to the previous experients, the ethanol vehicle was able to diffuse beyond the physiologically thinner urothelium in the mouse producing significant side-effects. The ethanol vehicle not only enhanced the amplitude of spontaneous activity but enhanced the release of ATP from the bladder lumen and the responses to purinergic, muscarinic and nerve-mediated stimulation. Although, non- receptor-mediated sensitivity accounted for the elevated responses

to purinergic and muscarinic stimulation. Pre-treatment with RTX produced some additional changes to bladder function which included bladder overactivity *in vivo*, elevated responses to KCl and receptor-mediated enhancements to purinergic and muscarinic stimulation *ex vivo*. There was also a reduction to distension induced ACh from the serosal surface of the bladder.

In conclusion, the ethanol vehicle for RTX has produced the majority of side effects associated with this treatment. Even though RTX treated animals had increased sensitivity on the receptor level to purinergic and muscarinic stimulation, EFS produced equally enhanced responses in the ethanol vehicle and RTX pre-treated animals that were persistent following normalization to KCl. This seems contradictory as non-receptor mediated sensitivity accounted for the elevation in the responses to purinergic and muscarinic stimulation in the ethanol vehicle pre-treated animals. This would suggest that other excitatory transmitters and neuropeptides are influencing neurotransmission which are predominantly mediated by ethanol. In addition to side effects produced by the ethanol vehicle, the increase in voiding frequency mediated by RTX may be related to enhanced purinergic and muscarinic receptor sensitivity including the reduction to distention induced ACh from the serosal side of the bladder.

Chapter 7:
General discussion

Intravesical therapy for Interstitial cystitis/bladder pain syndrome (IC/BPS) is usually indicated when more conservative measures have failed. Dimethyl sulphoxide (DMSO) is one of these intravesical treatments that is widely used and approved by the American Urological Association (AUA). However, since 2015, the European Urological Association (EUA) guidelines no longer recognise DMSO to be a treatment of value stating there is insufficient evidence (Hanno et al., 2011a, Hanno et al., 2015, Engeler et al., 2018). Although resiniferatoxin (RTX) and capsaicin are not approved for treating IC/BPS, there is potential benefit owing to its specific desensitizing action on transient receptor potential vanilloid-1 (TRPV1) channels found on the bladder afferent fibres (Szallasi and Blumberg, 1990, Mukerji et al., 2006a). Resiniferatoxin and capsaicin could potentially provide much-needed pain relief as these sensory fibres are upregulated by this condition (Liu et al., 2014). However, inconsistent results and painful side effects have restricted their use clinically.

Not much is known regarding how intravesical DMSO, RTX or capsaicin and their respective ethanol vehicles influence bladder function. The current studies addressed some of these knowledge gaps in the literature. For this thesis, bladder function after luminal treatment with DMSO, RTX and capsaicin and their respective ethanol vehicles (10% and 30% ethanol) was assessed immediately after its luminal application in the pig bladder while bladder function 24-hours after intravesical treatment with RTX and its ethanol vehicle was examined in the mouse bladder. This final discussion will compare the different models that were used, briefly discuss the complexities associated with IC/BPS including intravesical therapy, and specifically examine the effects and influence of these treatments on overall bladder function.

Bladder model comparison

To assess how luminal DMSO, RTX, capsaicin and ethanol affect bladder function and the probable implications it has for the IC/BPS patient, both pig and mouse models were utilized.

As mentioned in chapter two, pig bladder tissue is comparable to human bladder tissue in both structure and function. The tissue used in this model represents a full-thickness bladder wall complete with fully differentiated urothelium/lamina propria with glycosaminoglycan (GAG) layer, interstitial cells, nerves and detrusor. This model can assist in predicting an outcome of intravesical treatment for IC/BPS sufferers that have a normal appearing urothelium and GAG

layer. (Dixon et al., 1986, Tomaszewski et al., 2001). The luminal application of treatments in this model corresponds well to intravesical treatment in a clinical setting where the treatment solutions and their respective vehicles are placed onto the lumen of the bladder to dwell for 15-30mins (treatment dependent). Confounding factors include the inability to test the bladder as a whole unit, the lack of immune response and systemic influences (e.g. blood flow) to treatment. Some of these factors may inhibit the diffusion process and should be taken into consideration when interpreting the results. Tissue viability is also limited with this model, preventing examination of the long-term effects associated with treatment.

For the mouse model, both the *in vivo* and *ex vivo* effects of treatment can be observed. The mouse bladder represents the whole bladder, complete with fully differentiated urothelium, GAG layer, interstitial cells, nerves and detrusor. Similar to a clinical setting, intravesical treatments can be performed on an intact bladder with a fully functioning blood flow and immune system. Voiding behaviours can be observed before and after treatment to evaluate the physiological response to treatment, and the long-term effects of a particular treatment can also be examined in this model. For *ex vivo* studies, the bladder can be tested as a whole organ compared to bladder strips used in other models. Other advantages of this model are that neurogenic responses include a non-adrenergic, non-cholinergic (NANC) component while the urothelium is thin, these are two characteristics observed in patients with IC/BPS (Palea et al., 1993, Slobodov et al., 2004). However, care should still be taken when interpreting the results as there are still some species related variations associated with this model, even though out of all the rodents, its bladder is more comparable to the human (Andersson et al., 2011).

Urothelial alterations associated with IC/BPS – revisited

Ordinarily, the function of the urothelium is to act as an expandable barrier to urine and urinary solutes. This is physiologically achieved in part by hydrophobic plaques and uroplakins on the umbrella cells, tight junctions, adhesion proteins and the GAG layer (Lilly and Parsons, 1990, Hu et al., 2002, Apodaca, 2004, Zhang et al., 2005). In IC/BPS, some of these permeability promoting structures are altered (Parsons, 2002, Zhang et al., 2005, Slobodov et al., 2004, Jhang et al., 2016, Ong and Kuo, 2017). These alterations are hypothesized to add to the "leakiness" of the urothelium which is even more pronounced in patients with 'Hunner's ulcer" (Parsons et al., 1991, Erickson et al., 2000, Parsons et al., 2002). However, evidence for an intact GAG layer in IC/BPS is conflicting, as there are some reports of a normal appearing

intact GAG layer (Dixon et al., 1986, Nickel et al., 1993). Increased bladder permeability and noxious elements found in urine are speculated to stimulate sensory afferents and irritate the detrusor producing symptoms of IC/BPS (Parsons, 2011, Montalbetti et al., 2017).

Although IC/BPS can be categorized as ulcerating and non-ulcerating, it was observed by Messing and Stamey (1978) and Tomaszewski et al. (2001) that this was not predictive of symptoms. Urothelial thinning is a feature of IC/BPS, although the extent to which this occurs in the patient is highly variable and urinary pain has been associated with the degree of urothelium denudation (Tomaszewski et al., 2001). The variability of GAG expression and the state of the urothelium in IC/BPS patients may reflect disease progression ranging from mild in the early stages to severe, where pain becomes the more dominant symptom as time progresses (Parsons, 2002, Parsons, 2011).

Intravesical drug delivery and IC/BPS

Some of the difficulty associated with intravesical treatment is delivery of drug instillations beyond the barrier of the GAG and umbrella cell layer to the bladder wall as permeability is low in normal conditions (Lilly and Parsons, 1990, Parsons et al., 1994, GuhaSarkar and Banerjee, 2010). This may be of some advantage to IC/BPS patients with thin or a complete loss of urothelium. However, not all sufferers of IC/BPS have thinning of the urothelium, and they can also present with normal cystoscopic pathology and a morphologically normal GAG layer (Tomaszewski et al., 2001, Dixon et al., 1986). To complicate matters even further, for those suffering with more severe IC/BPS, deficits have been found in the GAG layer as well as increases in urinary excretion of GAGs. (Hurst et al., 1996, Wei et al., 2000, Lokeshwar et al., 2005) As pain seems to be associated with the state of the urothelium and intravesical drug delivery is determined by the urothelium and GAG layer, it would be prudent before proceeding with intravesical treatment, and after more conservative measures have failed, to perform a biopsy with cystoscopy to determine the appropriate treatment course to improve patient outcomes. For example, GAG layer replenishment may not be of benefit to patients with normal cystoscope findings, a fully functional GAG layer and intact urothelium. Although treatment with pentosanpolysulfate has shown more benefit than a placebo, it alleviated symptoms in a little less than 50% of participants; ulcers were present in 28% and pain present in 75% (Parsons and Mulholland, 1987). Another more recent study found no benefit compared to placebo. However, the participants were not officially diagnosed with IC/BPS nor by

cystoscopic findings and had milder symptoms (Nickel et al., 2015). These examples emphasise the point that due to the complexities and variability in the presentation of this condition one treatment does not suit all. Unfortunately at present, there is no non-invasive test to determine the condition of the urothelial barrier although some tests, e.g., antiproliferative factor (APF) in urine seems promising as a diagnostic tool (Kuo, 2014). As yet, there is no cure for IC/BPS, but biopsy along with cystoscopy could save time and money by identifying the state of the urothelium and barrier components.

Reported side effects associated with DMSO, RTX and capsaicin

Treatment with intravesical DMSO is beneficial in >50% of IC/BPS sufferers (Stewart et al., 1972, Perez-Marrero et al., 1988, Sant and LaRock, 1994). RTX and capsaicin are only used clinically in limited experimental studies for IC/BPS at this stage. However, the use of intravesical RTX is associated with some positive findings in IC/BPS studies while other studies have found no benefit which indicates that treatment with RTX is spurious at best (Chen et al., 2005b, Payne et al., 2005, Apostolidis et al., 2006). Capsaicin seems to have a more positive long-term outlook; however, the initial unpleasant side effects associated with its use can last up to two weeks (Cruz et al., 1997, Soontrapa et al., 2003). Capsaicin is not the only treatment with reported unpleasant side effects. Side effects associated with DMSO include an initial exacerbation of symptoms and a peculiar garlic body odour. (Sant, 1987, Sant and LaRock, 1994, Rossberger et al., 2005, Tutolo et al., 2017). In some DMSO studies, the drop-out rate due to side effects can be high, but the uncomfortable effects can improve with subsequent instillations (Barker et al., 1987, Tutolo et al., 2017). Sant (1987) also described DMSO with the addition of hydrocortisone in patients that don't respond favourably to the uncomfortable effects. Interestingly, Rossberger et al. (2005) observed that side effects following DMSO treatment were indistinguishable between ulcerating and non-ulcerating IC/BPS. Intravesical use of RTX has also associated with some uncomfortable side effects in a considerable number of patients (non-ulcerating IC/BPS), these are speculated to be milder than those produced by capsaicin (Chen et al., 2005b, Payne et al., 2005).

The rate of response to treatment with DMSO, RTX or capsaicin and the degree of unpleasant side effects associated with these treatments in IC/BPS patients may reflect the state of the urothelium and the effectiveness of the drug or vehicle that is used to overcome the bladders innate permeability barrier.

It is the primary goal of treatment with RTX or capsaicin to get these drugs to diffuse through the GAG layer and urothelium to reach their target which are the TRPV1 channels found on the sensory afferents beneath the urothelium and throughout the bladder. The action of RTX or capsaicin on these nerves depletes them of neurotransmitters which can potentially provide non-narcotic pain relief (Maggi et al., 1989a, Maggi et al., 1990b, Cruz, 1998, Ost et al., 2002). The primary target of DMSO is currently unknown, and there may not be any target given its status as a penetration enhancer (Williams and Barry, 2004). DMSO reportedly has an analgesic, anti-inflammatory, bacteriostatic and muscle relaxing effect (Jacob et al., 1964, Shirley et al., 1978).

The results of this study have clearly shown that DMSO and the vehicles for RTX and capsaicin (10% and 30% ethanol) produce the majority of unpleasant side effects that are associated with these treatments. Other groups have long suspected the irritability of ethanol in the bladder at both of these concentrations (Cruz et al., 1997, de Seze et al., 1998, Payne et al., 2005).

DMSO induced side effects in the pig bladder

Modifications to urothelium/lamina propria during treatment

During treatment with DMSO, the enhanced luminal release of adenosine 5'- triphosphate (ATP) and acetylcholine (ACh) and sloughing of the urothelial barrier and cells would allow easy passage of these mediators and also allow the contents of the bladder to come into contact with the sub-urothelial afferents and possibly the underlying structures (Birder and de Groat, 1992, Birder et al., 1997). Enhanced urothelial ATP release could potentially cause the reported increase in painful urination and ACh the increased frequency associated with treatment (Coutts et al., 1981, Yoshida et al., 2004, Peeker et al., 2000b). Birder et al. (1997) found that DMSO instilled in the whole rat bladder significantly increased the frequency of rhythmic bladder activity while filling and decreased the volume threshold for inducing micturition while it was in contact with the urothelium. The reason why some patients and not others tolerate it is uncertain and may very well reflect the overall state of the urothelium as pain, but not garlic odour was cited to be the determining factor for discontinued treatment (Tomaszewski et al., 2001, Tutolo et al., 2017).

Modifications to urothelium/lamina propria post treatment

After luminal pre-treatment, ATP release from the urothelium/lamina propria was undetected under basal and stretch conditions while ACh release was depressed in both conditions, most likely reflecting urothelial sloughing. As ATP and urothelially released ACh can be attributed to pain and urinary frequency, the regeneration of fresh urothelial tissues may contribute to the reported improvements in pain and urinary frequency after DMSO treatment (Ek et al., 1978, Peeker et al., 2000b). In some studies, DMSO has been found to be more effective at reducing pain, urinary frequency and improved the effectiveness of hydrodistension long term in patients with "Hunner's ulcer" (Peeker et al., 2000b, Tomoe, 2015). Perhaps the reason behind the reported efficacy associated with ulcerating IC/BPS is related to the degree of urothelial thinning or absence of the urothelium in these patients allowing DMSO to penetrate to the deeper layers of the bladder in contrast to the small urothelial fissures that have been seen in non-ulcerating IC/BPS (Peeker et al., 2000b, Tomaszewski et al., 2001, Slobodov et al., 2004). The effect of DMSO on the bladder sensory nerves has also been suspected to be the chief reason for its effectiveness (Barker et al., 1987). DMSO has been found to block the conduction of C-fibres in the cat sural nerve in a concentration-dependent manner at a minimum concentration of 9% that took 22-minutes to develop (Evans et al., 1993). DMSO at higher concentrations (>1%) is cytotoxic to rat lumbosacral DRG neurons (Birder et al., 1997). Assuming sensory nerve alteration produces the majority of long-term analgesic improvements in IC/BPS patients, a longer dwell time or adjustments to concentration or frequency of DMSO instillations may improve the outcome in non-ulcerating IC/BPS. However, the general urothelial thickness in relation to tolerance may determine which parameters that need to be adjusted.

Modifications to contractile activities post treatment

It is uncertain just how far DMSO had penetrated into the bladder wall given the full thickness of the urothelium for this study. However, parasympathetic nerve responses were enhanced at all frequencies immediately after pre-treatment, and enhanced contractile responses to muscarinic stimulation were only found in the urothelium/lamina propria. This is consistent with other studies that have recognised that exposure of the urothelium to DMSO can cause irritation and enhancement of parasympathetic pathways (Hohlbrugger and Lentsch, 1985, Birder and de Groat, 1992, Birder et al., 1997). The time course of increased sensitivity to

parasympathetic and muscarinic stimulation after treatment is uncertain, although, it may represent the reported exacerbation of symptoms after treatment which is generally short-lived (Parkin et al., 1997).

Ethanol-induced side effects in the pig and mouse bladder

The conclusion that the vehicle ethanol used in conjunction with RTX or capsaicin produces the majority of side effects was made apparent after observing that ethanol rather than the drugs produced more significant alterations to bladder function.

Modifications to urothelium/lamina propria during treatment

During treatment, ethanol at concentrations of 10% or 30% produced a concentration-dependent increase in urothelial ATP and significantly higher levels of LDH compared to its saline control while it was on the urothelium in the pig bladder. These enhancements remained unchanged in the presence of RTX or capsaicin. Histological analysis of the urothelium during treatment with 10% ethanol failed to demonstrate any change in urothelial thickness when compared to control tissues. However, the presence of LDH demonstrated that damage to the most superficial cells has occurred. The impact of treatment with 30% ethanol resulted in significant urothelial sloughing. The application of 30% ethanol would likely produce significantly more pain for the patient not only by the enhancement in ATP but also by the abolition of the urothelium allowing passage of noxious constituents to the deeper layers of the bladder (Coutts et al., 1981, Lilly and Parsons, 1990). The histological structure of the urothelium also remained unchanged by the presence of RTX or capsaicin.

Overall, these findings are consistent with patients experiencing mild discomfort associated with 10% ethanol compared to more severe discomfort associated with 30% ethanol (de Seze et al., 1998, Payne et al., 2005) Intriguingly, urothelial release of ACh in the presence of 30% ethanol was reduced in contrast to the vigorous increases found during treatment with DMSO and capsaicin. It has been suggested that non-neuronal ACh contributes to the regulation of the storage phase in the micturition cycle where antimuscarinics are thought to be the most effective (Yoshida et al., 2004). The reductions found in urothelial ACh during treatment with 30% ethanol may allow for improved dwell time during treatment when compared to DMSO and capsaicin.

Modifications to urothelium/lamina propria post treatment

When comparing urothelium/lamina propria responses in our two models immediately (pig) and 24-hours (mouse) after pre-treatment with ethanol, the following alterations remained unchanged in the presence of RTX or capsaicin. For the pig urothelium/lamina propria following pre-treatment with ethanol (10% or 30%), the release of ATP in basal and stretched conditions was reduced while ACh release was abolished in basal conditions. For whole mouse bladders, distension induced luminal ATP but not ACh was enhanced 24-hours after intravesical treatment with 10% ethanol. The disparity between the pig and mouse bladder after pre-treatment with 10% ethanol could reflect the difference in species such as transmitters and urothelial thickness or it could reflect the immediate effects vs. the effect 24-hours later (Phillips and Davies, 1980, Ehlert et al., 2007, Cheng et al., 2011b).

Modifications to contractile activities post treatment

10% ethanol

For pig urothelium/lamina propria and whole mouse bladder, 10% ethanol pre-treatment enhanced the amplitude of spontaneous activity immediately and 24-hours after pre-treatment with no further changes induced by the addition of RTX. From here on in, 10% ethanol pre-treatment in the pig bladder produced no more changes in response to the remaining bladder function tests. This is in contrast to the whole mouse bladder, in which the responses to parasympathetic, purinergic and muscarinic stimulation remained enhanced and unchanged 24-hours after RTX treatment. However, the enhanced response to purinergic and muscarinic stimulation was ascribed to non-receptor mediated mechanisms since normalisation to potassium chloride (KCl) reduced the responses so that they were no longer statistically different to those of control treated bladders. The enhanced sensitivity of the contractile components after ethanol treatment along with an increase in urothelial ATP release in the mouse bladder would likely produce pain and strong contractions upon urination. As the frequency of urination after this treatment was unaltered it suggests that urothelial ACh release under basal conditions remained unchanged. These findings, taken together would suggest that 10% ethanol can effectively penetrate the thinner urothelium to the deeper structures of the bladder but produces considerable side effects. As 10% ethanol does not appear to be a competent vehicle for drug delivery in bladders with a thicker urothelium, this might explain the conflicting results regarding the effectiveness of RTX in IC/BPS patients.

30% ethanol

Immediately following pre-treatment with 30% ethanol, the pig urothelium/lamina propria was less responsive to purinergic stimulation but more sensitive to non-receptor mediated stimulation (KCl) and muscarinic stimulation. The detrusor also became more sensitive to muscarinic stimulation and nerve-mediated contraction. These variations remained unchanged by the presence of capsaicin except for the detrusor response to muscarinic stimulation in which the enhancements originally made by ethanol were depressed. There are limited studies examining bladder function after the luminal application of 30% ethanol. However, the present findings would indicate that this concentration of ethanol was able to penetrate the thicker urothelium and access the bladder's deeper structures which would allow capsaicin to reach the sub-urothelial sensory nerves. As mentioned before, the reported severe side effects associated with intravesical 30% ethanol can take up to two weeks to diminish, and as a vehicle it would perhaps be more suited to IC/BPS patients with no abnormal urothelial observations (de Seze et al., 1998, Tomaszewski et al., 2001). Curiously, there have been reported improvements in control subjects' after intravesical treatment with 10% or 30% ethanol as the vehicle. Some authors have attributed these findings to the placebo effect but based on the results of this study, these changes to bladder function are most likely the result of the actions of ethanol (de Seze et al., 1998, Payne et al., 2005).

The additional effects of RTX and capsaicin.

It was the hope of this study to gain a better understanding of how RTX and capsaicin affected bladder functioning. However, the interference of ethanol has made this unclear. Nevertheless, some additional alterations to bladder function other than those produced by their vehicles were observed.

RTX

The addition of RTX produced a diminished response to ATP in the intact pig bladder tissue. The reduction in response was suggested to be due to an RTX-mediated interaction with urothelial purinergic receptors that has affected signalling as well as diffusion of nitric oxide (NO) from the urothelium/lamina propria through to the detrusor (see discussion, chapter five). For the whole mouse bladder, extra observations included an increase in voiding frequency after treatment, a decrease in distension induced release of serosal ACh and an increased

sensitivity to KCl. Tissues normalised to KCl to account for ethanol-induced non-receptor mediated sensitivity changes uncovered some unique receptor-mediated alterations facilitated by RTX. These alterations consisted of enhanced responses to purinergic and muscarinic receptor stimulation suggested to be due to the desensitization of afferent fibres which in turn release neuropeptides including prostaglandins enhancing the signal. The enhanced purinergic signal along with reductions in serosal ACh may be contributing to the increased voiding activity seen in these mice (see discussion, chapter six).

Capsaicin

Pig bladder treated with luminal capsaicin on the other hand, produced more urothelial ACh during treatment as well as enhanced ACh release from the urothelium/lamina propria under basal and stretched conditions after pre-treatment which was suggested to be due to neuropeptides like substance P (SP) and anticholinesterase activity (see discussion, chapter five). The only other observation attributed to capsaicin was a depression of detrusor muscarinic responses that were ordinarily enhanced by its ethanol vehicle which may demonstrate neuropeptide activity or the non-specific effects of capsaicin (discussed in chapter five).

Intravesical RTX and capsaicin with alternate delivery

It is not certain if these effects are the result of RTX or capsaicin alone or rather RTX or capsaicin with their vehicles working in synergy as RTX and capsaicin has been found to potentiate the effects of ethanol in other tissues (Abdel-Salam et al., 1995, Trevisani et al., 2002).

As it is becoming quite clear that ethanol alone produces numerous side effects, it is still unclear whether 50 nM RTX in a 10% ethanolic solution reaches its desired target in IC/BPS patients who may have an intact GAG layer and urothelium. Some success has been achieved with a reduction in pain three months after intravesical RTX treatment (Ham et al., 2012). However, in this case, RTX was instilled immediately following hydrodistension which stretches the urothelium of the bladder to capacity until urine begins to leak from the urethra. The improvement in pain symptoms was significant compared to hydrodistension alone. A plausible explanation for these results could be increased absorption of RTX through the stressed urothelium (Erickson et al., 2000). Improvements in symptoms have also been reported

in IC/BPS patients with a prolonged infusion of 10 nM RTX over a ten-day period (Lazzeri et al., 2004a).

Aware of the intense side effects associated with intravesical 30% ethanol and capsaicin, de Seze et al. (2006) trialled the use of a glucidic solvent as the vehicle for capsaicin. Although there were unpleasant side effects in a small proportion of subjects, consisting of pelvic pain during instillation with the solvent, none of the patients reported the pain lasting beyond the first day of treatment. Furthermore, no patient asked to withdraw from the study. Then again, the subjects for this study had neurogenic detrusor overactivity from spinal cord injuries and not IC/BPS.

Urothelial repair outcomes for IC/BPS patients after treatment with DMSO and ethanol

With regards to the urothelial sloughing that occurred in this study by treatment with DMSO and 30% ethanol, ordinarily, the urothelium is a slow cycling epithelium. However, injury can rapidly increase turnover, and it is uncertain how additional cytotoxic injury would affect cellular turnover in IC/BPS (Martin, 1972, Hicks, 1975, Hicks and Chowaniec, 1978). Generally, after the loss of urothelium, re-epithelialization begins as early as 4-hours after injury, and overall proliferation is maximal after 16-hours. Restoration to a fully differentiated intact urothelium is usually complete by day ten (de Boer et al., 1994, Bilbao et al., 2014). This time frame is similar to the one to two-week resolution of painful side effects for subjects with neurogenic detrusor overactivity that were treated with 30% ethanol as a vehicle control (de Seze et al., 1998).

The mechanisms by which urothelial wound healing occurs is complex but is thought to involve the synthesis and secretion of growth factors acting in an autocrine or paracrine way. Members of the epidermal growth factor (EGF) family, mainly, heparin-binding EGF (HB-EGF) have been found to promote urothelial cell growth after injury (Freeman et al., 1997, Daher et al., 2003). Moreover, HB-EGF is synthesized by the urothelium, detrusor and vascular tissue in the bladder (Freeman et al., 1997). Epidermal growth factor, on the other hand, has been found not to consistently stimulate the growth of the urothelium (Freeman et al., 1997, Daher et al., 2003). In normal urothelium, EGF receptors are mostly confined to the basal layers and not on the superficial cells.

However, in a pre-malignant or malignant urothelium, many EGF receptors are found on the superficial cells (Messing et al., 1987). Although EGF and HB-EGF are typically found in urine, their ratios become altered with IC/BPS compared to normal bladders with a lower HB-EGF and higher EGF content. These changes are thought to be due to the urothelial expression of APF (Keay et al., 1997, Keay et al., 2000). At this stage, it is uncertain how APF would impact the healing time after the use of such abrasive treatments and vehicles. As the detrusor and vascular tissue in the bladder have been found to synthesize HB-EGF, injury and removal of the urothelium after treatment with DMSO and 30% ethanol could potentially stimulate its expression promoting the growth of a healthier urothelium which may be of benefit (Freeman et al., 1997). In addition, it has been found in a small number of IC/BPS patients that percutaneous sacral third nerve root neurostimulation restored HB-EGF and APF to approximately normal values while at the same time improving urinary frequency and pain (Chai et al., 2000a). Furthermore, in response to stretch, the detrusor has been found to have an upregulated expression of HB-EGF mRNA while hydrodistension in IC/BPS patients has been found to increase HB-EGF toward normal control values and decrease APF which begin to approach pre-distension values two weeks after treatment (Park et al., 1998, Chai et al., 2000b).

Afferent mechanisms

DMSO and ethanol to some extent, but especially RTX and capsaicin have a well-known association with sensory nerves. Although afferent nerves were not tested in this study, there is evidence that activation of these nerves not only transmits impulses back to the central nervous system but also possess an efferent activity by the release of neuropeptides which can then modulate contractile mechanisms. (Maggi et al., 1984, Maggi et al., 1990b, Vasko et al., 1994, Daly et al., 2007)

These neuropeptides can act directly on the bladder tissues or indirectly by modifying other tissues that can influence bladder activity, they can also act on mast cells, immune cells, arterioles and endothelial cells producing warmth, plasma extravasation and hypersensitivity which is collectively termed “neurogenic inflammation”(Shirakawa et al., 1989, Richardson and Vasko, 2002, Templeman et al., 2003)

There is abundant evidence that has shown that the application of capsaicin and RTX promote the release of neuropeptides from capsaicin-sensitive nerves (Szallasi and Blumberg, 1989,

Maggi et al., 1990b, Ishigooka et al., 2000). In the rat bladder, the NK receptor was found to be primarily responsible for promoting capsaicin-induced extravasation (Eglezos et al., 1991). While there was no conclusive evidence of neurogenic inflammation in this study, some of the observed changes to bladder function such as alterations to mediator release and contractile activity may occur indirectly as a result of afferent nerve activation and subsequent release of neuropeptides.

Future investigations

Interstitial cystitis/bladder pain syndrome is a complex condition and treatment to manage symptoms can be perplexing. By itself, several questions and potential research avenues have been generated at the close of this thesis for future inquiry.

Epidermal growth factor receptors are mostly found in the basal cell layer of the urothelium in normal conditions while in premalignant and malignant conditions they have been found on the superficial cells (Messing et al., 1987). It has been suggested that the urothelium of patients with IC/BPS fails to differentiate correctly with a lack of umbrella cells and greater amounts of EGF in the urine would be granted uncomplicated access to the EGF receptors found in the basal layer (Slobodov et al., 2004, Messing et al., 1987, Keay et al., 1997). EGF in high concentrations (50 ng/mL) but not low concentrations (5 ng/mL) has been shown to alter the growth rate of cultured normal urothelial cells and more notably reduced their mitotic rate. The cells became larger, flatter and eventually died (Dubeau and Jones, 1987). The amount of EGF found in the urine of IC/BPS patients compared to control patients is ~17 ng/mL and ~8 ng/mL respectively (Keay et al., 1997). It would be thought-provoking to characterize EGF receptors in IC/BPS patients as well as examine the possible interactions EGF may have with APF. Also interesting is the mechanism behind lower APF and higher HB-EGF after hydrodistension in IC/BPS patients and how this would relate to EGF.

With the known difficulties associated with getting drugs beyond the urothelial layer, it would be useful to identify and catalogue the diffusion of penetration enhancers, along with potential side effects, in a variety of bladders ranging from young to old with varying urothelial thickness with a view to making treatment more efficacious which may ultimately open more possibilities for treatment.

It takes time for RTX to deplete sensory neurons of neuropeptides. In the rat, systemic RTX

has been found to be more efficient than capsaicin. One hour after treatment with RTX, xylene-induced neurogenic inflammation was reduced by 70% and was completely inhibited by day four after treatment (Szallasi et al., 1989). Our study has observed the effects of RTX immediately after and 24-hours after treatment. In the mouse, the analysis of our results was complicated by the vehicle which can take some time to resolve. It would be appealing to examine the effects seven days, two weeks and one month after treatment and to identify if RTX at a concentration of 50 nM is adequate knowing that it has a lower binding affinity to human and pig receptors when compared to the mouse and rat.

Finally, with regards to the afferent nerves, more evidence regarding neuropeptides, including their triggers and actions, could be helpful in clearing up some unknown complexities of the bladder. There is also evidence of interactions between TRPV1 channels and purinergic receptors in afferent nerves (Moriyama et al., 2003, Grundy et al., 2018b). Further exploration into this relationship and whether this exists in the urothelium may help to understand the role of ATP further.

Concluding remarks

The bladder is a complex organ that is not entirely understood and likewise so is its pathology in conditions such as IC/BPS. Overall, treatment with DMSO and the ethanol vehicles for RTX and capsaicin produce unpleasant side effects. It is still unclear what effects RTX and capsaicin had on the bladder as some may have been obscured by ethanol. Essentially, determining the diffusion of various vehicles through the bladder wall (including potential side effects) should be a major priority for urological study, and if appropriate, all pharmacological studies should use a vehicle control with a non-vehicle control to rule out interference. The reason DMSO and RTX work well in some and not other studies may be related to urothelial integrity. The ethanol vehicle for RTX and the concentration of RTX may not be sufficient for IC/BPS sufferers with an intact urothelium and more clinical studies are required. It is quite clear that the urothelium in IC/BPS is variable amongst individuals and a biopsy before determining intravesical treatment after conservative therapy has failed, could save time and money, and provide hope for the sufferer. At this stage, there is no cure for IC/BPS, and until more knowledge becomes available, symptoms need to be managed more effectively to improve quality of life and productivity. Capsaicin and RTX in addition to DMSO could potentially be vital tools in this process by helping to manage pain and other troublesome symptoms associated with IC/BPS.

In conclusion, intravesical treatment with DMSO, RTX and capsaicin produce unpleasant side-effects that are immediate and can be persisting. The respective vehicles for RTX and capsaicin (10% and 30% ethanol) produce most of the side-effects associated with their use. It is also uncertain whether 10% ethanol can diffuse past the intact urothelium to reach its sub-urothelial target.

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